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Serum Antibodies to Periodontal Pathogens and Anti-Malondialdehyde-Acetaldehyde: Potential Role in the Interrelationship Between the Periodontium and Rheumatoid Arthritis

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Serum antibodies to periodontal pathogens and anti-malondialdehyde-acetaldehyde: potential role in the interrelationship between the periodontium and rheumatoid arthritis

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ABSTRACT

SERUM ANTIBODIES TO PERIODONTAL PATHOGENS AND ANTI-MALONDIALDEHYDE-ACETALDEHYDE: POTENTIAL ROLE IN THE INTERRELATIONSHIP BETWEEN THE PERIODONTIUM AND RHEUMATOID ARTHRITIS

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Objectives: Study 1 – To quantify associations between anti-*Porphyromonas gingivalis*, anti-*Prevotella intermedia*, and anti-*Fusobacterium nucleatum* serum antibody concentrations and risk of developing rheumatoid arthritis (RA). Study 2 – To test if periodontal clinical measures, alveolar bone loss (ABL), and the aforementioned serum anti-bacterial antibody concentrations are associated with anti-malondialdehyde-acetaldehyde (MAA) serum antibody concentrations.

Methods: Study 1 – Serum samples pre- and post- RA diagnosis (n=214 cases, 210 matched controls) from the U.S. Department of Defense Serum Repository were utilized to compare timing of elevations of anti-bacterial antibody concentrations relative to RA diagnosis and associations between antibacterial antibody concentrations and RA autoantibodies. Study 2 – Periodontal clinical and ABL measurements were made on participants from five medical centers (n=284 RA cases, n=330 osteoarthritis controls). Serum anti-MAA and anti-bacterial antibody concentrations were quantified by ELISA. Both studies utilized linear regression models.

Results: Study 1 – No case-control divergence in serum anti-bacterial antibody concentrations was seen. In RA pre-diagnosis samples, anti-*P. intermedia* was significantly positively associated with rheumatoid factor and anti-CCP2. Study 2 – Moderate and high ABL in RA cases were significantly positively associated with IgG and IgM anti-MAA. Serum antibacterial antibody concentrations also displayed significant positive associations with anti-MAA antibodies.

Conclusions: These studies suggest a potential role of anti-*P. intermedia* in development of RA. ABL and antibodies to oral pathogens were associated with anti-MAA antibodies suggesting that MAA may play a role in the link between the periodontium and RA.

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LIST OF ABBREVIATIONS

AA	Acetaldehyde
ABL	alveolar bone loss
ACPA	anti-citrullinated protein antibodies
CK-13	cytokeratin 13
CRP	C-reactive protein
CV	coefficient of variation
DAS28	Disease Activity Score including 28-joint count
DMARDs	disease-modifying antirheumatic drugs
DoDSR	Department of Defense Serum Repository
ESR	erythrocyte sedimentation rate
GCF	gingival crevicular fluid
HRP	horse radish peroxidase
HSA	human serum albumin
IL	Interleukin
MAA	malondialdehyde-acetaldehyde
MAA-LDL	malondialdehyde-acetaldehyde-low-density lipoprotein
MDA	Malondialdehyde
MMP	matrix metalloproteinase
NETs	neutrophil extracellular traps
NSPT	nonsurgical periodontal treatment
OA	Osteoarthritis
OMA	outer membrane antigens
PAD	peptidylarginine deiminase
PD	Periodontitis
PGE2	prostaglandin E_2
PPAD	*P. gingivalis* peptidyl arginine deiminase
PTM	post-translational modification
RA	rheumatoid arthritis

RANKL	receptor activator of nuclear factor-KB ligand
RASPi	reactive aldehyde species inhibitor
RF	rheumatoid factor
ROS	reactive oxygen species
RgpB	*P. gingivalis* arginine gingipain
SE	shared epitope
TMB	Tetramethylbenzidine
TNF-α	tumor necrosis factor-α

CHAPTER 1: INTRODUCTION

Periodontitis (PD) is a chronic oral disease resulting from an interaction between the host immune response and a dysbiotic oral microbiota. PD is characterized by destruction of the periodontal ligament, gingival connective tissue, and alveolar bone and is a primary cause of tooth loss (1). This inflammatory disease has increasingly garnered attention not only for its oral health implications but also for its proposed systemic effects, notably in the pathogenesis of rheumatoid arthritis (RA) (2). RA, characterized by chronic synovial inflammation, joint damage, and systemic complications, shares intricate links with PD through shared inflammatory pathways, genetic factors, and environmental influences (3).

Studies have implicated *Porphyromonas gingivalis, Prevotella intermedia,* and *Fusobacterium nucleatum* as significant oral pathogens potentially bridging the gap between PD and RA (4-6). *P. gingivalis*, a keystone pathogen in PD, possesses a peptidylarginine deiminase (PAD) capable of citrullinating proteins, potentially leading to the production of anti-citrullinated protein antibodies (ACPA) that are highly specific to RA (7, 8). Furthermore, elevated anti–*P. gingivalis* antibodies in RA patients and a positive correlation with circulating ACPA levels were observed (9). *P. intermedia* is a periodontal pathogen that demonstrates potential involvement in the destruction of periodontal connective tissues and bone matrix through the upregulation of matrix metalloproteinases (MMP) production (10). An established association between *P. intermedia* infection and ACPA fine specificities further highlights its potential relevance to the RA-PD interrelationship (4). *F. nucleatum* is a periodontal pathogen found in periodontal diseases and endo-periodontal lesions (11, 12). While implicated in a wide spectrum of human diseases, *F. nucleatum* was found in the synovial fluid and dental plaque in an RA patient, suggesting possible bacterial translocation (13, 14). This intricate interplay between oral periodontal pathogens and the autoimmune responses observed in RA highlights the complex relationship between the periodontium and RA.

Moreover, oxidative stress has emerged as a contributing factor in RA pathogenesis (15), with highly immunogenic malondialdehyde-acetaldehyde (MAA) adducts produced during lipid peroxidation, resulting from the interaction between malondialdehyde (MDA) and acetaldehyde (AA), being implicated in various inflammatory diseases, including RA (16, 17). The association between anti-MAA antibodies and ACPA, as well as the increased presence of MAA-modified proteins in RA synovial tissues, suggests a potential link between these adducts and RA-related autoantibodies (18, 19). Oxidative stress also plays a role in PD either through direct excess of reactive oxygen species (ROS) activity/antioxidant deficiency or indirectly via the activation of redox-sensitive transcription factors leading to a pro-inflammatory state (20). Importantly, inflamed human periodontal tissues have been identified as a potential source of MAA-modified proteins, indicating a plausible connection between periodontal inflammation and systemic inflammatory responses demonstrated in RA (21).

As part of the dissertation presented herein, two studies were conducted to enhance our understanding of the complex interplay between the periodontium, specific oral pathogens, MAA-mediated immune responses, and the progression of RA. Study 1 aimed to 1) quantify the association between serum anti-*P. gingivalis* antibody concentrations and the risk of developing RA, and 2) quantify the associations among RA cases between anti-*P. gingivalis* serum antibody concentrations and RA-specific autoantibodies. Serum antibodies to *P. intermedia* and *F. nucleatum* also were assessed. Study 2 aimed to 1) evaluate if periodontal clinical measures, alveolar bone loss (ABL), and serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations were associated with anti-MAA serum autoantibody concentrations, and 2) determine whether associations of these measures with anti-MAA serum antibody differed between RA cases and osteoarthritis (OA) controls.

CHAPTER 2: LITERATURE REVIEW

Background on Rheumatoid Arthritis

RA is a chronic autoimmune disease of unknown origin. RA is characterized by a progressive inflammation of affected joints resulting in cartilage damage, bone destruction and, ultimately, disability (22-24). It is prevalent in 0.4% to 1.3% of the population and influenced by sex, age, and social residence in addition to other genetic and environmental factors. Women are two- to three-times more likely to be affected than men, disease incidence peaks during the sixth decade of life, and an increased frequency of RA is detected in urban versus rural areas (22, 23). The joints most commonly affected are the hands, feet and knees (25).

Currently, the etiology of RA has not been fully elucidated but available evidence suggests that multiple biologic pathways likely conspire leading to a common clinical presentation (26). It is been reported that the joint inflammation in RA is initiated and maintained by a multifaceted interaction between inflammatory cells consisting of T cells, B cells, neutrophils, dendritic cells, and macrophages, with fibroblasts and osteoclasts (26-28). During NETosis, neutrophil extracellular traps (NETs) are formed and PADs are activated (29). PADs function to citrullinate proteins in the joints and in other tissues, resulting in a self-sustaining localized immune response.

Immune complexes in the joints can signal and activate immune cells to release chemokines and cytokines that elicit and/or amplify local inflammation leading to articular destruction (30). Pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), are responsible for dysregulating the balance between the formation and destruction of cartilage and bone matrix within the joint. Continuous immune cell activation results in an overproduction of inflammatory cytokines and acts to sustain chronic inflammatory conditions in the joint characterized by proliferation of the synovial membrane and pannus generation (31, 32). Frequently, only a few joints are affected at onset; however, the inflammation

characteristically progresses to affect multiple joints in addition to extra-articular sites in later stages, such as rheumatoid nodules and pulmonary manifestations (33, 34).

Anomalies in the immune response lead to the development of autoantibodies, most notably rheumatoid factors (RF) and ACPA. These autoantibodies are primarily detected in serum and synovial fluid of RA patients. RFs were the first type of autoantibodies identified in RA and serve to recognize the Fc-tail of immunoglobulin IgG. Although RFs are predominantly comprised of the IgM isotype, other isotypes of RF have been researched, including both IgG and IgA (35, 36). RFs are not specific to RA as these can be detected in other systemic conditions, including leprosy, syphilis, pulmonary tuberculosis, chronic liver disease, sarcoidosis, SLE and Sjogren's disease (37-43). In addition, circulating RFs can be detected in healthy patient populations (usually in low concentrations), and IgM levels elevate with age while IgG decreases with age. Although RFs lack specificity for RA, the autoantibody test can be considered helpful in diagnostics and is used widely in clinical care (44, 45).

Additionally, the assessment of circulating ACPA plays a prominent role in RA diagnosis and have a disease specificity in RA approaching 95%. ACPAs, most commonly measured using a commericial anti-cyclic citrullinated peptide (anti-CCP) antibody assay, recognize citrulline-containing motifs, a ubiquitous post-translational modification (PTM). During the process of citrullination, arginine is transformed into citrulline by a calcium-dependent PAD enzyme. Proteins that can be citrullinated include fibrin, collagen, vimentin, and histone (46), among others. Cigarette smoking, a primary risk factor in RA, appears to upregulate PAD expression and increases the amount of citrullination present in lung tissues (47). Thus, inflamed lung tissues have been suggested as a possible site of early tolerance loss in RA characterized by the local expression of ACPA.

ACPAs are detectable for years prior to RA diagnosis, persist in patients with established RA and are prevalent in 50-70% of RA patients (48). ACPAs, like RFs, include a broad spectrum of antibody isotypes such as IgM, IgG and IgA (36, 49) and recognize multiple citrullinated

antigens, including α-enolase, vimentin, and histone (50-54). Anti-α-enolase antibodies were found in high levels in patients with RA versus controls (55, 56). Also detected in the joints of RA patients, citrullinated vimentin has been shown to induce ACPA production and result in bone loss through increased osteoclastogenesis (57, 58). Lastly, recent evidence suggests that citrullinated histones are correlated with NETs to drive RA pathogenesis (59). Distinct ACPA recognition patterns have been speculated to provide information on disease phenotype and the increase in activation of ACPA expressing B-cells (60). Currently, patients are assessed for both RF and ACPA to enhance diagnostic accuracy, particularly in the early stages of RA (61).

Malondialdehyde–Acetaldehyde Adducts and Rheumatoid Arthritis

Another product of interest is MAA. ROS can result in lipid peroxidation, leading to the formation of protein adducts and cell damage (19). MDA, formed by oxidative stress and lipid peroxidation, can react with AA, resulting from cigarette smoke and alcohol, to form MAA (62-65). MAA adducts have been demonstrated in multiple inflammatory disease conditions including cardiovascular, liver and lung disease (62, 66-68). Chronic alcohol consumption is associated with MAA adduct formation, which induces antibody and T-cell proliferative responses speculated to play a role in the pathogenesis of alcohol-related liver injury (69). MDA protein modifications have also been found in RA synovium during active RA disease periods and serum IgG anti-MDA levels correlated with disease activity measured by DAS28-ESR and with serum levels of pro-inflammatory cytokines, such as TNF-α (70). MDA levels were found to be higher in patients with RA versus patients with osteoarthritis (OA) and healthy controls (71). Both anti-MDA and anti-MAA antibodies have been reported to induce osteoclast maturation and bone erosion during RA (72), suggesting that the antibodies may play a direct pathogenic role in disease. However, limited research has been conducted regarding the relationship of MAA with the apparent links between PD and RA.

To detect the presence of MAA in RA synovium and its role in RA pathogenesis, one study compared the synovial tissues from patients with RA and patients with OA (18). Increased expression of MAA adducts was observed in RA synovial tissues compared to OA synovial tissues. In addition, serum anti-MAA antibody isotypes were increased in RA cases versus controls. Interestingly, MAA adducts co-localized with the citrullinated proteins in the RA synovial tissues but did not in the tissues derived from OA controls. This study suggests that MAA modifications could influence the immune response in RA patients (18).

A study by Mikuls et al. examined serum autoantibodies to MAA prior to RA diagnosis to determine if these autoantibodies might also (and autoantigens by implication) play a role in disease development. The authors analyzed serum samples from the U.S. Department of Defense Serum Repository from military personnel before and after their RA diagnosis along with paired samples from individuals free of RA. Within 2.3 and 3.0 years prior to RA diagnosis, IgA and IgG anti-MAA antibody isotypes, respectively, diverged significantly from controls without RA. This divergence for anti-MAA antibody occurred later, more proximate to disease onset, than observed for ACPA and differences in anti-MAA antibody expression were most striking among ACPA positive RA cases. The longitudinal study suggests that MAA formation and anti-MAA immune response, along with ACPA, play a role in progressing towards clinically-detectable RA (73).

Risk Factors for Rheumatoid Arthritis

It has become evident that a combination of genetics, epigenetics and environmental factors collectively predispose individuals to aberrant immune responses following an injury or infectious event, rendering the individual more susceptible to the onset of RA. Mucosal surfaces, including lungs, gut and periodontium, have shown to be affected in patients with RA (23, 74, 75). In terms of genetics, the abnormal response to citrullinated epitopes seen in patients with RA is firmly correlated with a specific set of polymorphisms of HLA-DRB1 (76). One twin study

suggested that RA displays a genetic predisposition as ACPA-positive and ACPA-negative RA have a similar heritability of approximately 66%, despite the fact that ACPA-negative RA is less prevalent (77). When comparing identical to non-identical twins, there was approximately ~60% RA disease heritability, defined as the phenotypic variance attributed solely to genetics (78). Risk factors for the development of RA have been reported to include age, cigarette smoking and other inhalant biohazards, obesity, female sex hormones, PD, and perhaps select infections (22, 79-81). Additionally, Lundstrom et al. demonstrated a significant interaction between HLA-DRB1 shared epitope (SE) alleles and smoking in the development of ACPA-positive RA, illustrating the complex interplay between environmental factors and genetic predisposition (82). Among these factors, the link between PD and RA development is especially fascinating.

Background on Periodontitis

Both RA and PD result from a complex chronic inflammatory response and can exhibit periodic flares of disease activity (83). While RA is considered an autoimmune disease, PD has an infectious etiology resulting from dysbiosis of the oral microbiota. PD may begin as early as childhood and adolescence but predominates during early adulthood. In adults over the age of 30, the prevalence of PD is estimated at 42%. Due to the cumulative effect of plaque and the chronic nature of the disease, PD prevalence jumps to 59.8% in the age group of 65 years and older (84).

PD is clinically characterized by bleeding or suppuration upon probing due to inflammation from pocket formation and loss of supporting tissues (gingiva, periodontal ligament, and alveolar bone) (85). The disease develops over time through dental plaque and calculus accumulation and bacterial dysbiosis, resulting in eventual tooth loss and considerably impacting one's quality of life.

A group of anaerobic, Gram-negative bacteria are strongly associated with PD and play a significant role in the pathological changes in the periodontium. The 'red complex' group consists of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*. Colonization of the

gingival sulcus by a combination of the red complex with *Aggregatibacter actinomycetemcomitans* can disrupt the healthy composition of the bacterial community, comprised chiefly by Gram-positive aerobes, to the Gram-negative anaerobes through a process called microbiota shift (2, 86).

The immune system initiates its defense in PD by sending neutrophils to the lesion site, promoted by chemical mediators including cytokines such as TNF-α, chemokines, and prostaglandin E_2 (PGE_2) (2, 87). Also, there is an accumulation of microbial and host-derived molecular patterns called pathogen-associated molecular patterns and danger-associated molecular patterns (88), and degrading enzymes such as collagenase, elastase, and gelatinase (87). The function of this initial immune response includes elimination of microbes followed by clearance of the resulting cellular debris by monocytes and macrophages. In an efficient and healthy immune system, there is no or minimal tissue damage to the surrounding tooth and the bacterial invasion is effectively removed (89).

However, if the bacterial species continue to proliferate or in the presence of a defective/altered immune response, the acute periodontal inflammation transforms to chronic inflammation and additional mediators are produced. These molecular events become the main culprit of destruction to the periodontium, acting similarly to a self-perpetuating autoimmune condition (90). In an established lesion, the pathogens are resistant to attack due to an enrichment of dysbiotic biofilm and the persistent immune response results in chronic inflammation (86). The damage towards the periodontium is less from the bacteria themselves, but heavily attributed to the dysregulation of the innate immune response against the bacteria (91).

Malondialdehyde–Acetaldehyde and Periodontitis

Limited studies have been performed that researched the relationship between MAA and PD. Salivary IgA autoantibodies to MAA-modified low density lipoproteins (LDL) were shown to cross-react with *P. gingivalis*, suggesting that the pathogen may act via antibody cross-reaction

and molecular mimicry with oxidized LDL antigens (92). In addition, salivary IgA antibodies to MAA-LDL were associated with moderate periodontal probing depths of 4-5 mm. A significant association also was observed between moderate probing depths and salivary IgA levels to known periodontal pathogens *P. gingivalis, P. intermedia,* and *A. actinomycetemcomitans* (93). A pivotal study to evaluate the association between MAA and PD was performed by Bright et al. (21). They evaluated gingival biopsies of patients with healthy, mild, and moderate PD and triple stained with antibodies against MAA adducts, citrullinated, and carbamylated proteins. Although highly similar to citrullination where arginine is modified to citrulline, carbamylation differs as lysine is modified to homocitrulline (94). Healthy gingival tissue revealed negligible staining results. Mild PD was positive for carbamylated, citrullinated, and MAA-modified proteins with greater staining intensity for all three found in moderate PD. This study demonstrated the presence of all three PTM proteins in close relation to the inflammatory cell infiltrate in inflamed periodontal tissues (21).

Risk Factors for Periodontitis

A complex interplay of subgingival microbes, host immune response, and environmental and genetic factors contribute to the development and severity of PD (91). Risk factors for periodontal disease include age, sex, race, income, education, and smoking (95). Several studies report associations between PD and numerous systemic inflammatory conditions, including arthritis, type 2 diabetes mellitus, and atherosclerosis (96-98). Regarding RA, one study reported an estimated 4% prevalence of RA in a PD test group, approximately six times greater than those found in the control group (99). With respect to the reciprocal relationship, an analysis of NHANES data by de Pablo et al. noted the prevalence of PD is approximately two-times greater in RA patients than non-RA patients and tooth loss is more common in RA patients (100). Similarly, another study reported that RA was associated with a higher likelihood of tooth loss, but no association with PD was seen after adjusting for confounding factors (101). On the other

hand, an increased risk for PD has been displayed in patients with RA (2, 102-105) while treatment for PD in RA patients demonstrate positive effects in decreasing both PD and RA disease activity (106, 107).

The Relationship between Rheumatoid Arthritis and Periodontitis

Despite having varying etiologies, both PD and RA are multifactorial diseases characterized by chronic inflammatory reactions and share multiple features. First, both have an elevated infiltration of immune response cells, including neutrophils, monocytes, and T and B lymphocytes. Second, both are characterized by an increased release of pro-inflammatory mediators such as TNF-α, IL-1, IL-6, and matrix-degrading enzymes (MMPs, cathepsins). Third, both share enhanced activation of the factor nuclear kappa B ligand (RANKL) pathway, stimulated by mediators released from immune cells, and followed by osteoclast differentiation that ultimately leads to the destruction of adjacent bone (2, 108). Anti-inflammatory mediators, IL-10 and transforming growth factors-β, are suppressed and an increase in C-reactive protein (CRP) level is present in the plasma, signaling systemic inflammation in both PD and RA (2).

The relationship between PD and RA was first described with a “two-hit” model theory by Golub et al. The first “hit” is the increased number of anaerobic microorganisms and their antigens in the periodontal environment. Following this theory, PD’s damaging events are triggered through increased generation of bone-resorptive cytokines and tissue-destructive proteinases. The second “hit” consists of a systemic disease, such as RA, resulting in an overall increase of biomarkers for systemic inflammation. This further stimulates immune cells in the periodontium to produce MMPs and RANKL which can destroy periodontal tissues, resembling the cytokine-driven osteoclast activation and bone resorption seen in RA (109).

In addition to sharing histopathologic features, PD and RA share common risk factors. PD shares smoking and genetic risk factors with RA. The SE-coding HLA-DRB1 allele, which more avidly binds to citrullinated peptides (versus native arginine-containing residues), connects

RA and PD (110). Bone destruction in RA and supporting alveolar bone resorption in PD have both been associated with the HLA-DRB1 allele (111). Furthermore, the allele has been implicated in the production of ACPA in response to *P. gingivalis* oral infection in an animal study (112).

Porphyromonas gingivalis and Rheumatoid Arthritis

P. gingivalis is recognized as a keystone pathogen in the pathogenesis of PD and is responsible for enriching the microbial community's virulence, even if present in low levels (113). One meta-analysis demonstrated that patients exposed to *P.gingivalis* had a higher odds ratio for RA (114). Uniquely, it is the only prokaryote that can express a functional bacterial PAD enzyme (often termed PPAD) as a primary virulence factor, thus serving as a microbe of interest in its role with RA and PD. The discovery of *P. gingivalis* PAD led to the hypothesis that *P. gingivalis* PAD-mediated protein citrullination at affected periodontal sites could launch a sequence of events that culminate in the generation of ACPAs and, eventually, in the clinical manifestation of RA (91).

Citrullination by host-derived PAD may be augmented by bacterial-derived PADs and amplify the production of ACPAs before the induction of RA, thus having a pivotal role in its pathogenesis (115). *P. gingivalis* can also promote NET formation as another source of citrullinated self-antigens (116). *P. gingivalis* has the ability to promote the generation of pro-inflammatory cytokines, such as IL-6 and IL-1β, through immune cells (117). Oral infections with *P. gingivalis* prior to RA onset can induce a Th17 cell response that can accelerate arthritis development (118).

In a study by Maresz to determine the significance of *P. gingivalis* PAD, a wild-type *P. gingivalis* was compared with a PAD-deficient *P.gingivalis* or *P. intermedia*. Results suggest that infections with wild-type *P.gingivalis* significantly stimulate the production of autoantibodies to type II collagen and citrullinated epitopes compared to the PAD-deficient microbes (119). This

further demonstrates a relationship of *P. gingivalis* between RA that appears to be mediated at least in part through this PAD-like enzyme.

The prevalence of *P. gingivalis* in the oral microbiota of RA patients was associated with the presence of ACPA in a study by Hitchon et al (120). Elevated levels of citrullinated proteins in RA patients can lead to a decreased tolerance to endogenous citrullinated antigens resulting in autoimmune responses (120). Moreover, an increase in citrullinated proteins is associated with a more aggressive form of RA along with earlier onset of bone destruction (2, 121). Mikuls et al. demonstrated that ACPA concentrations were associated with the presence of antibodies against *P. gingivalis* and were elevated among RA patients with PD (102). In the same study cohort, greater ABL was associated with higher ACPA concentrations (122).

Fisher et al. evaluated the risk of RA and pre-RA autoimmunity in a southern European population in relation to smoking, *P. gingivalis* arginine gingipain antibodies (anti-RgpB), and citrullinated PPAD peptides. Approximately 103 cases and 309 controls were analyzed, with median time to diagnosis in pre-RA cases of 7 years. Results show that smoking was significantly associated with an increased risk of RA and former smoking was associated with ACPA positivity in pre-RA cases. However, no association with pre-RA was found with anti-RgpB and citrullinated PPAD peptides when compared to controls. In patients progressing to RA, in contrast to aforementioned literature, this lack of antibody response suggests *P. gingivalis* may not account for the relation between PD and RA (123).

A study by Johansson investigated whether elevated anti-*P. gingivalis* antibody levels appear before the onset of RA symptoms and ACPA response. Anti-RgpB and anti-CPP3 antibodies were evaluated to explore the hypothesis that *P. gingivalis* triggers a breach of immune tolerance, preceding the detection of ACPA, and resulting in the progression to clinical RA. The study included 251 cases and 198 controls in a Swedish population with median time pre-dating RA symptoms of 5.2 years. In contrast to the previous study mentioned (123), Johansson and colleagues demonstrated high antibody concentrations against anti-RgpB in individuals

developing RA, specifically in ACPA-positive RA. RA development and antibody response of anti-RgpB were independent from smoking history or the presences of HLA-DRB1 SE. Enhanced levels of anti-CPP3 antibodies were also found in individuals with pre-clinical as well as those with established RA. Interestingly, there was no association observed between anti-RgpB and anti-CPP3 antibodies. The authors suggested that the anti-CPP3 antibody response should be categorized as a classic ACPA-specific response rather than a *P. gingivalis* antibody-specific response. This study supports a relationship between *P. gingivalis* and RA by demonstrating increased concentrations of anti-*P. gingivalis* antibodies in RA patients compared to controls, detectable years before developing symptoms (8). With opposing results from the two studies, additional research is needed to further explore whether antibody concentrations to the oral pathogen *P. gingivalis* may be increased prior to onset of RA symptoms and linked to the development of RA.

Prevotella Intermedia and *Fusobacterium nucleatum* and Rheumatoid Arthritis

In addition to the extensive research focused on *P. gingivalis* in understanding the pathogenesis of RA, *P. intermedia* has also been proposed to be involved in the progression of RA, albeit through a different mechanism. *P. intermedia* is a gram-negative anaerobic pathogenic bacteria and a part of the orange complex in PD (124). A study by Schwenzer et al. measured the antibody response to a novel citrullinated peptide, cytokeratin 13 (CK-13), in gingival crevicular fluid (GCF) and evaluated other ACPAs to citrullinated peptides in GCF and periodontal tissues in patients with PD. The authors found anti-cCK13-1 and anti-tenascin-C antibodies levels were correlated to each other and were associated with antibodies to *P. intermedia* but not to anti-RgpB. Since *P. intermedia* does not express a PAD, the authors suggested that mechanisms underpinning the induction of ACPA fine specificities must differ from *P. gingivalis* (4). Their proposed mechanism was degradation of NETs by nucleases from *P. intermedia* which could release PADs (29) and increase periodontal pathogenicity (125). Antibody levels to other

citrullinated peptides (α-enolase, fibrinogen β, and vimentin) were correlated with each other and linked to smoking and shared epitope. Thus, within the study, two distinct groups of ACPA fine specificities were identified: 1) linkage to smoking and shared epitope and 2) linkage of anti-tenascin-C and cCK13-1 to infection with *P. intermedia* (4).

Antibodies to periodontal pathogens in RA patients in remission was investigated by Kimura et al. They evaluated synovitis, as detected by ultrasound, and its association with periodontal pathogens and established biomarkers of RA. Greater *P. intermedia* antibody titer was observed in active RA patients and RA patients in clinical remission with subclinical synovitis, detected by ultrasound, compared to RA patients in clinical remission without subclinical synovitis. An association of *P. intermedia* antibody titer and disease activity of RA, specifically synovitis, was proposed. The mechanism proposed by the authors is activation of macrophages by *P. intermedia* which initiates production of IL-6 and TNF-α, inflammatory cytokines that play a role in periodontal and joint destruction. As more than half of RA patients in remission experience subclinical synovitis, the finding that persistent synovitis may be associated with *P. intermedia* leads to additional hypotheses regarding whether periodontal therapy could improve the disease activity of RA (126).

Therapy targeting one disease and evaluating its effectiveness to treat the other concomitant condition is another area of interest with PD and RA. One example is a study by Rinaudo-Gaujous et al. utilizing infliximab therapy (a monoclonal anti-TNF-α antibody) to treat RA and evaluating its effect on levels of *P. gingivalis* and *P. intermedia* antibodies. Overall, patients with RA were more frequently positive with respect to antibodies to *P. gingivalis* and *P. intermedia* compared to healthy patients. After six months of infliximab treatment, a slight increase of antibody levels of *P. gingivalis* and *P. intermedia* were seen. An association was seen between anti-*P. intermedia* antibody concentrations and IgM RF concentrations but not with anti-CCP2 concentrations. On the other hand, anti-*P. gingivalis* antibody concentrations were correlated with anti-CCP2 concentrations and not with IgM RF, affirming the potential role of *P.*

gingivalis-mediated citrullination in RA. With these differences in antibody correlations, the data suggests *P. intermedia* produces a unique immunologic response in patients with RA compared to *P. gingivalis* (127).

Contrary to these two studies, other research was unable to demonstrate a relationship between *P. intermedia* and RA. A study by Martínez-Rivera et al. focused on salivary ammonium levels and the relationship between *P. gingivalis*, *P. intermedia*, and *Tannerella forsythia* presence and RA disease activity. Metabolism of arginine and urea by oral bacteria generates ammonium in saliva and the authors wanted to determine the amount contributed by bacterial PAD. A lack of association was found between *P. intermedia* and RA disease activity and *P. intermedia* in relation to total ammonium levels. When compared to the other bacterial strains, *P. intermedia* appeared to contribute less to the production of ammonia nor was *P. intermedia* a contributor to RA disease by altering PAD activity, suggesting its influence is through a different process (128).

Continuing the search for a causal link, Sato *et al*. introduced *P. gingivalis* and *P. intermedia* into the gut microbiota and evaluated changes to collagen-induced arthritis mice. After administering the *P. intermedia* infectious agent, no significant increase in *P. intermedia* antibody, Il-17, or ACPA production were seen. Although the composition of the gut microbiota was altered to some extent by *P. intermedia*, albeit less than by *P. gingivalis*, infection with *P. intermedia* had minimal effect on arthritis progression compared to the significant effect seen with *P. gingivalis*-infected mice (129). These findings were consistent with a previous mouse study that reported *P. intermedia* produces a weaker host inflammatory response, when compared to *P. gingivalis*, but that its role in RA should not be overlooked (119).

While also a part of the orange complex and a major periodontal pathogen (124), limited studies exist evaluating anti-*F. nucleatum* antibody concentrations in RA. One study analyzed saliva samples of early RA patients and found microbiota rich in *F. nucleatum* when compared to healthy controls and proposed the oral microbiota may be useful in risk assessments for early

onset of RA (5). In looking at subgingival biofilm of RA patients, *F. nucleatum* was found in higher concentrations in anti-CCP antibody positive patients with RA versus controls, though this finding was not statistically significant (130). In a separate study, *F. nucleatum* was found in the synovial fluid of RA patients derived from both native and prosthetic joints. Identical clones of the bacteria were found in at least one patient's plaque sample, and it was proposed that *F. nucleatum* can translocate from the oral cavity to the synovial cavity (14). When compared to *P. gingivalis* and *P. intermedia*, potential mechanisms linking *F. nucleatum* and RA risk remain poorly understood.

The Effects of Periodontal Treatment on Rheumatoid Arthritis and RA Treatment on Periodontitis

RA treatment consists of various pharmacological approaches utilizing nonsteroidal anti-inflammatory drugs, glucocorticoids, and synthetic and biological disease-modifying antirheumatic drugs (DMARDs). These pharmaceutical approaches, focused on early and effective DMARD initiation, serve to reduce pain, inflammation, and alter the progression of joint deterioration to enhance the quality of life. In a recent systematic review, patients with RA and PD taking synthetic and biologic DMARDs demonstrated a reduction in probing depths, clinical attachment loss, bleeding on probing and gingival index (131).

Limited therapeutics exists for MAA. One study examined an expanded autoantibody profile (including RF, ACPA and anti-MAA) to identify patients that would highly benefit from biologic therapy for individualized RA treatment (132). Additionally, methotrexate treatment, a dihydrofolate reductase inhibitor, resulted in a 6-fold reduction in MAA-adduct formation and scavenged free radicals formed during MAA-adduct formation; the authors proposed that these mechanisms can subsequently decrease inflammation and tissue damage (133).

Compared to RA patients with normal levels of TNF-α, RA patients with increased circulating levels of TNF-α can exhibit exacerbated periodontal parameters, such as bleeding

upon probing, probing depths and clinical periodontal attachment loss (134). Additionally, RA disease activity correlated with serum levels of IL-6, TNF-α and CRP and might influence BOP (135). One study found TNF blockers used during treatment of patients with RA resulted in overall decline of biochemical markers of PD, including IL-1 and IL-8, in the GCF of established PD patients (122). In another study, systemic anti-TNF-α treatment led to decreases in periodontal inflammation and TNF-α concentration in the GCF of patients suffering with both arthritis and PD (136). Targeting TNF-α as a treatment modality appears to benefit RA, an FDA-approved indication, and PD simultaneously. The treatment regimen of DMARDs including anti-TNF improved clinical attachment level in RA patients compared to the untreated control group (137). Due to their demonstrated anti-inflammatory effects in the oral cavity, biological DMARD treatment may be suggested as an adjunctive therapy for prevention or treatment of PD in patients with RA.

Mechanical debridement, also known as scaling and root planing, is the main treatment of choice in PD patients. Scaling and root planing aims to remove calculus and alter the microbial flora to reduce inflammation in affected periodontal sites. Adjunctive pharmacological treatment is not commonly used except in aggressive forms of PD where debridement, with the additional use of antibiotics, may improve clinical outcomes of treatment (138). Nonsurgical periodontal treatment (NSPT) resulted in a decline in RA Disease Activity Score including 28-joint count (DAS28) scores and erythrocyte sedimentation rate (ESR) in RA patients whereas CRP, IL-6 and TNF-α levels were unaffected (139). A similar finding was noted in a recent meta-analysis that described a reduction in DAS28-ESR and ESR with periodontal therapy (140). Additional studies have reported short-term decreases in ESR (141, 142) and CRP levels post-NSPT (143, 144). Notably, a study by Zhao et al., revealed that patients with RA and PD exhibited elevated levels of CRP, ACPA, ESR and DAS28 compared to patients with RA only. However, one month after NSPT, those with both RA and PD experienced significant reductions in these rheumatologic parameters (145).

In a recent systematic review by Inchingolo et al., the authors discussed other studies that have reported minimal to no effect of NSPT on RA (131). One study found that performing NSPT twice a year for 24 months did not affect DAS28-ESR but did decrease the prevalence of the red complex pathogens subgingivally (146). In separate studies, NSPT did not significantly affect the levels of CRP (147) or ESR at 2-3 month follow-ups (148). While NSPT may not affect treatment of RA activity, it can improve general health in terms of psychological, physical, and social aspects (149). Furthermore, NSPT may influence the levels of serum autoantibodies associated with RA, particularly ACPA and RF, although these outcomes also vary across studies. NSPT has been shown to decrease ACPA (145, 150) and RF levels in patients with RA (151). Paradoxically, studies have shown an increase in ACPA and RF levels following NSPT (152).

Mechanistically, NSPT seems to modulate pro-inflammatory cytokines and mediators in various biological fluids, including serum, GCF, synovial fluid, and saliva. In serum, NSPT has been shown to reduce levels of TNF-α, IL-6, RANKL, carbamylated protein, NETs, and the neuroendocrine hormone prolactin (142, 150, 153-156). In GCF, reduction in the levels of TNF-α, IL-1β, IL-6, MMP-8, PGE2, tissue plasminogen activator, and prolactin were also noted as a result of NSPT (154, 156-159). RA patients undergoing NSPT demonstrate a reduction of prolactin in synovial fluid (156). Furthermore, NSPT has been shown to decrease RANKL levels in saliva (160). These results collectively suggest that periodontal therapy may contribute to reduced RA activity in RA with comorbid PD.

Summary

The complex relationship between RA and PD cannot be overlooked with the growing body of evidence linking the two diseases. The two diseases share similar inflammatory pathways and various risk factors, such as age, environment, smoking, socioeconomic status, and genetics. Therapeutic modalities in treating one chronic disease may benefit the other.

Evidence suggests that the foundational PD periodontal pathogen, *P. gingivalis*, uniquely impacts PD and RA through citrullination. *P. intermedia* has been shown to induce ACPA through a different mechanism from *P. gingivalis* as it does not express a PAD and *F. nucleatum* was more abundant in the salivary microbiota of early RA patients versus healthy controls. Furthermore, inflamed gingival tissues can be a source of MAA-modified protein and, possibly, influence RA pathogenesis. Therefore, our hypothesis for the first study was that anti-*P. gingivalis* serum antibody levels would be higher among individuals who subsequently developed RA versus controls and that anti-*P. gingivalis* antibody concentrations would be associated with preclinical levels of anti-CCP, ACPA fine specificities and RF concentrations. Anti-*P. intermedia* and anti-*F. nucleatum* also were evaluated to determine whether associations were specific to *P. gingivalis* or alternatively related to a broader dysbiosis present in PD. For our second study, we hypothesized that periodontal clinical measures, including ABL and serum antibodies to select oral pathogens (*P. gingivalis*, anti-*P. intermedia* and anti-*F. nucleatum*) were associated with serum anti-MAA autoantibody concentrations and evaluated whether these associations were different between RA cases versus OA controls.

CHAPTER 3: SERUM ANTIBODIES TO PERIODONTAL PATHOGENS PRIOR TO RHEUMATOID ARTHRITIS DIAGNOSIS: A CASE-CONTROL STUDY

Abstract

Objectives: 1) To quantify the association between anti-*Porphyromonas gingivalis* serum antibody concentrations and the risk of developing rheumatoid arthritis (RA), and 2) to quantify the associations among RA cases between anti-*P. gingivalis* serum antibody concentrations and RA-specific autoantibodies. Additional anti-bacterial antibodies evaluated included anti-*Fusobacterium nucleatum* and anti-*Prevotella intermedia*.

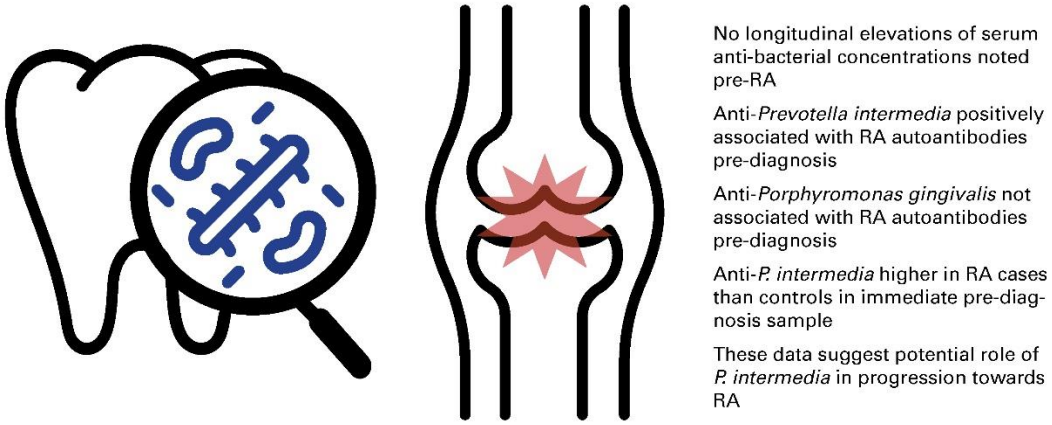
Methods: Serum samples were acquired pre- and post- RA diagnosis from the U.S. Department of Defense Serum Repository (n = 214 cases, 210 matched controls). Using separate mixed-models, the timing of elevations of anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations relative to RA diagnosis were compared in RA cases versus controls. Associations were determined between serum anti-CCP2, ACPA fine specificities (vimentin, histone, and alpha-enolase), and IgA, IgG, and IgM RF in pre-RA diagnosis samples and anti-bacterial antibodies using mixed-effects linear regression models.

Results: No compelling evidence of case-control divergence in serum anti-*P. gingivalis*, anti-*F. nucleatum*, and anti-*P. intermedia* was observed. Among RA cases, including all pre-diagnosis serum samples, anti-*P. intermedia* was significantly positively associated with anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA RF ($p<0.001$), IgG RF ($p=0.049$), and IgM RF ($p=0.004$), while anti-*P. gingivalis* and anti-*F. nucleatum* were not.

Conclusions: No longitudinal elevations of anti-bacterial serum antibody concentrations were observed in RA patients prior to RA diagnosis compared to controls. However, anti-*P. intermedia*

displayed significant associations with RA autoantibody concentrations prior to RA diagnosis, suggesting a potential role of this organism in progression towards clinically-detectable RA.

Key Words: rheumatoid arthritis, periodontitis, ACPA, rheumatoid factor, *Porphyromonas gingivalis*, *Prevotella intermedia*



Graphical Abstract

Lee JA, Mikuls TR, Deane KD, Sayles HR, Thiele GM, Edison JD, Wagner BD, Feser ML, Moss LK, Kelmenson LB, Robinson WH, Payne JB. Serum antibodies to periodontal pathogens prior to rheumatoid arthritis diagnosis: A case-control study. Semin Arthritis Rheum. 2023 Apr;59:152176. doi: 10.1016/j.semarthrit.2023.152176. Epub 2023 Feb 11. PMID: 36812865; PMCID: PMC10243205.

Introduction

It has been hypothesized that rheumatoid arthritis (RA) may be initiated in mucosal tissues, including the periodontium (74). Periodontitis (PD) is a biofilm-driven inflammatory

disease of the soft and hard tissues in the oral cavity resulting from an interaction between the host immune response and a dysbiotic oral microbiota, ultimately leading to tooth loss (1). Over the past few decades, there has been an increased awareness of the relationship between RA and PD. Both diseases share similar inflammatory pathways and risk factors (3). Several studies have demonstrated PD as a risk factor for RA (161-163). It has been speculated that this relationship may be mediated through the oral periodontal pathogen, *Porphyromonas gingivalis* (164).

P. gingivalis is a gram-negative anaerobe recognized as a keystone pathogen in the pathogenesis of PD (165, 166). Uniquely, it is the only prokaryote that can express a functional bacterial peptidyl arginine deiminase (PAD) enzyme (often termed PPAD) as a primary virulence factor, thus serving as a microbe of interest in its role with RA and PD (167). The discovery of *P. gingivalis* PAD led to the hypothesis that *P. gingivalis* PAD-mediated protein citrullination at affected periodontal sites can launch a sequence of events that culminate in the generation of anti-citrullinated protein antibodies (ACPAs) and, eventually, in the clinical manifestation of RA (6). However, while *P. gingivalis* is a periodontal pathogen implicated in RA pathogenesis, other bacterial species involved in PD, such as *Prevotella intermedia* and *Fusobacterium nucleatum*, may also influence development of RA (4, 5).

We hypothesized that circulating concentrations of antibody to *P. gingivalis* would be higher in samples from individuals later developing RA compared to controls. Anti-bacterial serologies may be used as a surrogate of exposure to periodontal pathogens and we have previously reported associations between serum antibody to *P. gingivalis* and RA-related autoantibody expression among patients without clinically apparent RA, but with a higher risk of future disease (168). Moreover, we postulated that, among those with RA, anti-*P. gingivalis* antibodies would be associated with the presence of RA-related autoantibodies prior to diagnosis. The purpose of this study was to: 1) quantify the association between anti-*P. gingivalis* serum antibody concentrations and the risk of developing RA, and 2) quantify the associations among

RA cases between anti-*P. gingivalis* serum antibody concentrations and RA-specific autoantibodies. Additional anti-bacterial antibodies evaluated included anti-*P. intermedia* and anti-*F. nucleatum* to determine whether associations observed were specific to *P. gingivalis* or related to a broader dysbiosis that may be observed in PD.

Methods

Patient population

Study participants consisted of military personnel participating in the U.S. Department of Defense Serum Repository (DoDSR). Since 1996, DoDSR has been collecting serum samples to observe health history in the military population and further understand the risks of deployment concerning subsequent injuries or chronic illnesses (169).

Active-duty personnel with ≥2 RA diagnostic codes (≥1 from a rheumatologist) were screened from the military's electronic medical records (170). The records were further examined to obtain the date of diagnosis and fulfillment of the 1987 American College of Rheumatology classification criteria (171). Serum samples were acquired prior to and after RA diagnosis for up to four samples per case, a minimum of two samples and up to three samples from pre-diagnosis, collected at different time points, and one sample from post-diagnosis. This study utilized 214 RA cases who received a diagnosis of RA between 1995 and 2012. Out of these cases, 212 met the 1987 RA classification criteria and the other two cases were diagnosed by a board-certified rheumatologist. These RA cases were chosen because there was a clear date of RA diagnosis recorded, adequate information to evaluate the clinical course of their RA after diagnosis, and two or more pre-diagnosis and one post-diagnosis serum samples with adequate volumes available for analysis.

Controls were selected and matched to each case based on age (at time of RA diagnosis for their matched cases), sex, ethnicity, enlistment region, and duration of sample storage. Exclusions for the controls were a history of RA or other inflammatory arthritis (170).

Four of these controls were subsequently excluded due to insufficient information available to exclude inflammatory arthritis, leaving a total of 210 controls evaluable for the analysis. These cases and controls were included in earlier DoDSR studies by our group (73, 170).

Clinical data collected included: age at time of diagnosis, sex, ethnicity, smoking status (those with missing data after chart review were imputed as never smokers), sample collection timing relative to RA diagnosis, follow-up time and RA medications received post-RA diagnosis, radiographic erosions, and number of samples tested (170).

Serum autoantibody assays

ACPA was determined using a commercially-available second-generation anti-CCP2 ELISA (Diastat, Axis-Shield Diagnostics, Dundee, Scotland); CCP2 positivity was based on the manufacturer's recommendation at a level of > 5 U/ml. Serum samples also were evaluated for 26 specific ACPAs using a bead-based multiplex antigen array that measures antibody reactivity to a panel of putative citrullinated auto-antigens (172). To reduce the chance of false discovery, analyses of antigen-specific ACPAs were limited to antibodies targeting citrullinated forms of vimentin, alpha-enolase, and histone, which are autoantigens consistently implicated in RA pathogenesis (57, 59, 96). IgA rheumatoid factor (RF), IgG RF, and IgM RF concentrations (IU/ml) were determined using ELISA (Inova Diagnostics, San Diego, CA). RF positivity was based on concentrations for each isotype (IgA RF, IgG RF, and IgM RF) determined to be present in < 2% of controls.

Serum bacterial antibodies

Serum concentrations of IgG antibodies to outer membrane antigens (OMA) of *P gingivalis, P. intermedia,* and *F. nucleatum* were measured by ELISA, as described in a previous publication from our group (168).

Ethical considerations

The Institutional Review Boards approved the study protocol at the DoDSR, Walter Reed National Military Medical Center, and the University of Colorado Multiple Institutional Review Board.

Statistical analyses

Participant characteristics were compared between RA and control groups using chi-square tests, exact chi-square tests, t-tests, or Wilcoxon rank sum tests as necessary. Autoantibodies and bacterial antibodies were log (base 2) transformed for all analyses. The primary analysis investigated the associations between anti-*P. gingivalis* serum antibodies and RA diagnosis (i.e., case status). Anti-*P. intermedia* and anti-*F. nucleatum* were evaluated to determine whether associations observed were specific to *P. gingivalis* or conversely related to a broader dysbiosis observed in PD. Initial analyses compared anti-bacterial antibody concentrations and biomarkers (i.e., anti-CCP2, ACPA fine specificities targeting vimentin, histone and alpha-enolase, and RF isotypes) between groups in the pre-RA diagnosis sample that was closest to diagnosis, and the post-RA diagnosis sample using Wilcoxon rank sum tests. The timing of elevations in anti-bacterial concentrations were evaluated in RA cases versus controls in a manner previously described (73, 170). Briefly, we used mixed models for each bacterial concentration with a continuous time effect modeled using B-splines and assuming a multivariate normal distribution for random subject intercepts and slopes. At each month prior to diagnosis, we compared autoantibody concentrations to identify the first instance where concentrations differed significantly ($p < 0.05$) between cases and controls. These multiple comparisons were using a stepdown Holm-simulated method. Correlations between anti-bacterial antibody concentrations were evaluated by Pearson correlation coefficient.

Secondary analyses examined potential associations between anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations and biomarkers (i.e., anti-CCP2, ACPA fine specificities targeting vimentin, histone and alpha-enolase, and RF isotypes) within

the RA group. These analyses were completed using both unadjusted and adjusted mixed-effects linear regression models with either RF or ACPA as the dependent variable, a fixed effect for each of the anti-bacterial antibodies in turn, and random subject intercepts. The adjusted models also included terms for age, sex, and smoking status.

All analyses were performed utilizing SAS v9.4 (SAS Institute, Cary, NC).

Results

Participant characteristics and autoantibody values

Patient characteristics and median autoantibody concentrations of the participants are shown in Table 1. RA cases were slightly more likely than controls to be ever smokers (32% vs. 23%, p=0.05); however, when analysis was limited to non-missing data, RA cases and controls did not differ with respect to ever smokers (p=0.15). Higher median serum concentrations of anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA, IgG and IgM RF isotypes were observed for the immediate/closest pre-diagnosis sample and post-RA diagnosis sample in RA cases versus controls (p<0.001). Likewise, anti-CCP2 positivity and IgA, IgG, and IgM RF isotype positivity were significantly higher for the immediate/closest pre-diagnosis sample and post-RA diagnosis sample in RA cases versus controls (p<0.001) (Table 1).

Serum anti-bacterial antibodies in RA cases versus controls

Median anti-*P. gingivalis* serum antibody concentrations were not significantly different between RA cases and controls with respect to the immediate/closest pre-diagnosis or post-diagnosis samples (Table 2). Median anti-*P. intermedia* serum antibody concentrations were significantly higher in RA cases than controls for the immediate/closest pre-diagnosis sample (p=0.008), but not in the post-diagnosis sample. In contrast, median anti-*F. nucleatum* serum

antibody concentrations were lower in RA cases than controls in the immediate/closest pre-diagnosis sample (p=0.045) but did not differ in post-diagnosis samples.

Association between pre-RA diagnosis serum anti-bacterial antibody concentrations and future RA case status

Temporal relationships of anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* serum antibody concentrations with RA cases and controls are shown in Figure 1. No evidence of case-control divergence in anti-*P. gingivalis* and anti-*P. intermedia* was observed during the pre-RA diagnosis period. Anti-*F. nucleatum* displayed evidence of slight case-control divergence at 13 years, 7 months prior to diagnosis, with the controls having higher anti-bacterial antibodies than the cases, but values reconverged and were not significantly different at all later time points. Correlations among the anti-bacterial serum antibody concentrations were moderately strong and positive (r=0.46-0.66; data not shown).

Autoantibody concentrations among RA cases and associations with anti-bacterial antibodies

In analyses limited to RA cases, using data from all pre-diagnosis observations, anti-*P. gingivalis* and anti-*F. nucleatum* serum antibody concentrations were not significantly associated with any of the RA autoantibodies in either unadjusted analyses or in multivariable models adjusted for age, sex, and smoking status (Table 3).

However, higher anti-*P. intermedia* serum antibody concentrations were significantly associated with higher concentrations of anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA RF autoantibodies (p<0.001) in both unadjusted and adjusted analyses. Anti-*P. intermedia* serum antibody concentrations were also significantly associated with IgG RF (p=0.047, 0.049) and IgM RF (p=0.003, 0.004) for unadjusted and adjusted values, respectively.

Discussion

This study shows serum anti-*P. intermedia* antibodies demonstrated significant associations with anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA, IgG, and IgM RF autoantibody concentrations prior to RA diagnosis even after adjusting for age, sex, and smoking. In contrast, anti-*P. gingivalis* and anti-*F. nucleatum* serum antibody concentrations were not significantly associated with RA autoantibodies. Additionally, no longitudinal elevations of anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* serum antibody concentrations were observed in RA patients prior to a diagnosis of RA compared to controls.

Prior studies have evaluated serum anti-*P. gingivalis* antibody concentrations in association with pre-RA case status (8, 123, 173). Fisher et al. evaluated a southern European population prior to the onset of RA and reported the association between smoking and antibodies to *P. gingivalis* arginine gingipain (RgpB), and citrullinated PPAD peptides with the risk of RA and pre-RA autoimmunity (123). Median timing from blood sampling to diagnosis in pre-RA cases was seven years. Their results showed that smoking was significantly associated with an increased risk of RA before clinical onset of disease and former smoking was associated with ACPA positivity in pre-RA cases. Antibodies to RgpB and PPAD peptides were not associated with risk of RA or with pre-RA autoimmunity. Similar to our study, *P. gingivalis* antibody was not associated with pre-RA autoimmunity or risk of RA and the authors suggested this organism may not play a role in the association between PD and RA in this cohort (123).

A study by Johansson analyzed a Northern Swedish population and investigated whether anti-*P. gingivalis* antibody levels pre-dated the onset of RA symptoms and ACPA production (8). The median time blood samples pre-dated RA symptoms was approximately five years. In contrast to the Fisher et al. study (123), their data demonstrated significantly increased anti-RgpB

IgG levels in pre-symptomatic patients and in RA patients compared with controls. Enhanced levels of antibodies to a citrullinated PPAD peptide (anti-CPP3) were also found in both pre-symptomatic and RA individuals. Interestingly, no significant association was noted between anti-RgpB and anti-CPP3 antibodies. This study supported a relationship between *P. gingivalis* and RA by demonstrating increased concentrations of anti-*P. gingivalis* antibodies in RA patients compared to controls, detectable years before symptom development (8).

Manoil et al. measured serum IgG antibodies against selected periodontal pathogens, including *P. gingivalis*, to determine whether they were associated with early symptoms or RA development (173). This study did not find an association between serum IgG titers against individual periodontal pathogens and specific preclinical phases of RA development. However, the authors found an association between cumulative IgG titers against periodontal pathogens and ACPA-positivity. These data suggest that synergy among periodontal pathogens, rather than specific bacterial associations, may be associated with ACPA development (173).

Our results may differ from prior reports given differences in the populations studied. In the present study, antibody to *P. gingivalis* was directed against outer membrane antigens (OMA), rather than only to specific *P. gingivalis* virulence factors seen in the other two studies (8, 123). Also, the majority of our study population was male and consisted of active United States military personnel compared to individuals residing in Northern (8) or Southern Europe (123). Furthermore, the mean age of the RA cases in the European studies were around 50 years old and had a high percentage of ever smokers, ranging from 59% (123) to 67% (8), while our RA participants averaged 37 years old and had lower smoking prevalence of 32%. With the differences in age at disease onset, our younger cohort could suggest a high genetic burden for RA. That high genetic risk could potentially attenuate the importance of environmental factors in this population, such as smoking and bacterial infection leading to PD (102). We did not

determine HLA-SE in the current study, although a previous publication by our group found no evidence of an interaction of PD with HLA-DRB1 SE positivity (102).

In our previous study, relationship of *P. gingivalis* with RA autoantibodies in individuals at "high risk" for RA was examined (168). Patients were considered autoantibody positive with one or more positive autoantibody tests and high-risk individuals were either ACPA-positive or were positive on two or more RF assays. No patients satisfied the 1987 American College of Rheumatology RA classification criteria (171). Anti-*P. gingivalis* concentrations were higher in both the high-risk and autoantibody positive groups than in the autoantibody negative group. There were no differences between groups with respect to anti-*P. intermedia* or anti-*F. nucleatum*. The majority of this cohort was slightly older and predominantly female when compared to our younger, male population and could account for the different associations with serum anti-bacterial antibodies (168). These contrasting conclusions suggest additional research is needed to further explore whether antibody concentrations to the pathogen *P. gingivalis* may be increased prior to onset of RA symptoms and linked to the development of RA.

Although *P. gingivalis* is the most studied periodontal microorganism in the pathogenesis of RA, it has been suggested that *P. intermedia* may also play a role in RA progression, albeit by a different mechanism. A study by Schwenzer et al. suggested that, since *P. intermedia* does not express a PAD, its ability to induce ACPA differs from *P. gingivalis* (4) potentially through a mechanism whereby degradation of neutrophil extracellular traps (NETs) by nucleases from *P. intermedia* leads to the release of PADs (29) and increases the pathogenicity of this organism (125). Kimura et al. (126) evaluated synovitis and its association with periodontal pathogens and established biomarkers of RA. Greater *P. intermedia* antibody titer was observed in active RA patients and RA patients in clinical remission with subclinical synovitis, detected by ultrasound, compared to RA patients in clinical remission without subclinical synovitis. An association of *P. intermedia* antibody titer and disease activity of RA, specifically synovitis, was proposed. The

mechanism suggested by the authors is activation of macrophages by *P. intermedia* which initiates production of IL-6 and TNF-α, inflammatory cytokines that play a role in periodontal and joint destruction. Of note, Scher et al. reported that *Prevotella* and *Leptotrichia* species were the only characteristic taxa in the oral microbiota in the new-onset RA group irrespective of PD status and were completely absent in the oral microbiota of controls (174). While other investigators were unable to demonstrate a relationship between *P. intermedia* and RA (128, 129), our study observed a strong association with anti-CCP2, certain ACPA specificities as well as several isotypes of RF and highlights a need to further explore the potential role of *P. intermedia* in RA pathogenesis and, in particular, the generation of these RA-related autoantibodies.

Limited studies exist evaluating anti-*F. nucleatum* antibody concentrations with RA. One study analyzed saliva samples of early RA patients and found microbiota rich in *F. nucleatum* when compared to healthy controls and proposed the oral microbiota may be useful in detecting risk assessment for early onset of RA (5). In looking at subgingival biofilm of RA patients, *F. nucleatum* was found in higher concentrations in aCCP-positive patients with RA versus controls, though this finding was not statistically significant (130). In a separate study, *F. nucleatum* was found in the synovial fluid of RA patients derived from both native and prosthetic joints. Identical clones of the bacteria were found in the same patient's plaque sample, and it was proposed that *F. nucleatum* can translocate from the oral cavity to the synovial cavity (14). In contrast, our data does not provide compelling evidence to support a role of *F. nucleatum* in RA development. In contrast to prior reports, our results demonstrated only a slight case-control divergence of anti-*F. nucleatum* prior to RA diagnosis; however, controls had initially higher concentrations that reconverged to no longer be statistically significant than concentrations in RA cases. When compared to *P. gingivalis and P. intermedia*, potential mechanisms linking *F. nucleatum* and RA risk remain poorly understood.

There are limitations in this study. The participants were military personnel with a relatively high proportion of men to women (52% vs. 48%, respectively) and a younger age of RA onset (37 years old). Thus, these results may not be generalizable to other RA populations (175). A majority of RA cases utilized methotrexate and/or biologics (88% and 74%, respectively), which could have impacted these results. Furthermore, there were only 112 control patient samples available for post-RA diagnosis evaluations. The lack of a difference in anti-*P. intermedia* concentrations in RA cases versus controls post-RA diagnosis needs to be interpreted with caution in light of this smaller sample size available for analysis. In addition, most of the pre-RA serum samples were collected within 5 years of diagnosis, which could have limited our ability to detect earlier differences in anti-bacterial or autoantibody elevations (73). In future studies, more frequent serum sample collection over more extended time periods would provide an even more comprehensive look at the autoantibody and anti-bacterial responses potentially leading to RA onset. PD status was not determined in this study and, therefore, we were unable to associate periodontal status with the patients' systemic response against the periodontal pathogens investigated. Moreover, the taxa could exert a local response without triggering a serum IgG response; therefore, null associations should be carefully considered. Finally, future studies also should focus on the plethora of inflammatory reactions occurring in the gingival tissues that have the potential to stimulate autoantibody production associated with RA.

Conclusion

In conclusion, no longitudinal elevations of serum anti-bacterial antibody concentrations were observed in RA patients prior to a diagnosis of RA compared to controls. However, anti-*P. intermedia* displayed a significant association with RA autoantibody concentrations prior to RA diagnosis, suggesting a potential role of this organism in progression towards clinically-detectable RA.

Data availability statement

Data requests can be made to the authors although use is restricted based on Department of Defense guidelines.

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Disclosure statement

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The identification of specific products or scientific instrumentation is considered an integral part of the scientific endeavor and does not constitute endorsement or implied endorsement on the part of the author(s), DoD, or any component agency. The views expressed in this article are those of the author(s) and do not necessarily reflect the official policy of the Department of Defense or the U.S. Government.

Table 1

Patient characteristics and autoantibody values

Characteristic	RA Cases n=214	Controls n=210	p-value
Age at time of diagnosis, mean (SD)	36.8 (7.9)	36.7 (8.0)	0.89[a1]
Sex, n (%)			
Female	102 (48)	101 (48)	0.93[a2]
Male	112 (52)	109 (52)	
Ethnicity, n (%)[b]			
White	123 (59)	122 (60)	
Black	58 (28)	55 (27)	
Hispanic	18 (9)	18 (9)	1.00[a3]
Asian	5 (2)	5 (2)	
American Indian	4 (2)	4 (2)	
Other	1 (0)	1 (0)	
Ever Smoker, n (%)[c]	68 (32)	49 (23)	0.05[a2]
Anti-CCP2, U/ml, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	59 (2, 216)	0.3 (0.1, 1.0)	<0.001[a4]
Post diagnosis sample	52 (3, 204)	0.4 (0.1, 0.9)	<0.001[a4]
Anti-CCP2, U/ml, n (% positive)[d]			
Immediate / closest pre-diagnosis sample	152 (72)	3 (1)	<0.001[a3]
Post diagnosis sample	153 (72)	0 (0)	<0.001[a3]
ACPA against vimentin, MFI, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	288 (93, 1725)	60 (47, 79)	<0.001[a4]
Post diagnosis sample	397 (86, 1849)	53 (47, 68)	<0.001[a4]
ACPA against histone, MFI, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	591 (133, 2563)	91 (71, 126)	<0.001[a4]
Post diagnosis sample	546 (122, 2478)	77 (62, 108)	<0.001[a4]
ACPA against alpha-enolase, MFI, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	310 (108, 4431)	82 (67, 103)	<0.001[a4]
Post diagnosis sample	406 (112, 3640)	78 (65, 98)	<0.001[a4]
IgA RF, IU/ml, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	5.8 (2.1, 27.1)	1.3 (0.9, 2.0)	<0.001[a4]
Post diagnosis sample	5.7 (1.8, 29.3)	1.2 (0.9, 2.0)	<0.001[a4]
IgA RF, IU/ml, n (% positive)[d]			
Immediate / closest pre-diagnosis sample	86 (41)	3 (1)	<0.001[a3]
Post diagnosis sample	86 (40)	4 (4)	<0.001[a3]
IgG RF, IU/ml, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	6.4 (4.7, 11.6)	4.5 (3.5, 5.7)	<0.001[a4]
Post diagnosis sample	6.7 (4.2, 11.7)	4.4 (3.3, 5.8)	<0.001[a4]
IgG RF, IU/ml, n (% positive)[d]			
Immediate / closest pre-diagnosis sample	40 (19)	5 (2)	<0.001[a3]
Post diagnosis sample	35 (16)	1 (1)	<0.001[a3]

IgM RF, IU/ml, median (IQ range)[d] Immediate / closest pre-diagnosis sample Post diagnosis sample	 30 (8, 105) 30 (8, 105)	 3.8 (2.1, 7.0) 3.6 (2.2, 7.7)	 <0.001[a4] <0.001[a4]
IgM RF, IU/ml, n (% positive)[d] Immediate / closest pre-diagnosis sample Post diagnosis sample	 112 (53) 112 (53)	 7 (3) 4 (4)	 <0.001[a3] <0.001[a3]
RA medications (Ever Used), n (%)			
Methotrexate	187 (88)	-	-
Anti-TNF inhibitor	157 (74)	-	-
Radiographic erosions, n (%)	95 (45)	-	-
Number of samples tested, per individual, n (%) 2 3 4	 0 (0) 3 (1) 211 (99)	 1 (0) 102 (49) 107 (51)	-
Span of pre-RA samples in years, mean (SD)	-5.1 (5.7)	-	-
Span, oldest to newest sample, in years, mean (SD)[e]	12.8 (5.2)	12.2 (4.9)	0.30[a1]

ACPA = anti-citrullinated protein antibodies
MFI = mean fluorescent intensity
[a1]t-test. [a2]Pearson chi-square test. [a3]Exact Pearson chi-square test. [a4]Wilcoxon rank-sum test.
[b]Each ethnicity group was missing values for 5 cases and 5 controls.
[c]Data missing regarding 'ever smoking' in 5 cases and 89 controls (imputed as never smokers); when analysis limited to non-missing data, ever smoking observed in 33% of cases and 41% of controls (p=0.15).
[d]Immediate pre-diagnosis samples available for 212 cases and 207 controls; post-diagnosis samples available for 214 cases and 112 controls.
[e]Among 214 cases and 112 controls with a post diagnosis/index date sample.

Table 2

Serum anti-bacterial antibodies in RA cases versus controls

Serum anti-bacterial antibodies	RA Cases n=214	Controls n=210	p-value
Anti-*P. gingivalis*, ug/ml, median (IQ range)[a]			
Immediate / closest pre-diagnosis sample	47 (29, 83)	50 (30, 86)	0.633
Post diagnosis sample	50 (31, 84)	53 (30, 87)	0.913
Anti-*P. intermedia*, ug/ml, median (IQ range)[a]			
Immediate / closest pre-diagnosis sample	331 (258, 412)	313 (220, 379)	0.008
Post diagnosis sample	372 (289, 445)	371 (289, 423)	0.404
Anti-*F. nucleatum*, ug/ml, median (IQ range)[a]			
Immediate / closest pre-diagnosis sample	57 (34, 86)	61 (39, 105)	0.045
Post diagnosis sample	57 (33, 96)	67 (42, 97)	0.192

[a]Immediate pre-diagnosis samples available for 212 cases and 207 controls; post-diagnosis samples available for 214 cases and 112 controls

Table 3

Associations of all pre-diagnosis autoantibody sample concentrations among RA cases with serum anti-bacterial antibodies

	Unadjusted		Adjusted[a]	
Dependent Variable	**Anti-*P. gingivalis* coefficient (95% CI)**	**p-value**	**Anti-*P. gingivalis* coefficient (95% CI)**	**p-value**
Anti-CCP2	0.101 (-0.274, 0.475)	0.597	0.105 (-0.274, 0.484)	0.586
ACPA against vimentin	0.020 (-0.180, 0.220)	0.845	0.032 (-0.170, 0.233)	0.758
ACPA against histone	0.168 (-0.027, 0.363)	0.092	0.173 (-0.024, 0.371)	0.085
ACPA against alpha-enolase	0.073 (-0.132, 0.277)	0.485	0.094 (-0.111, 0.299)	0.367
IgA RF	-0.066 (-0.244, 0.112)	0.465	-0.060 (-0.239, 0.119)	0.510
IgG RF	0.038 (-0.057, 0.133)	0.434	0.040 (-0.056, 0.137)	0.411
IgM RF	0.019 (-0.158, 0.195)	0.835	0.030 (-0.144, 0.205)	0.733
Dependent Variable	**Anti-*P. intermedia* coefficient (95% CI)**	**p-value**	**Anti-*P. intermedia* coefficient (95% CI)**	**p-value**
Anti-CCP2	1.838 (1.210, 2.466)	<0.001	1.869 (1.235, 2.503)	<0.001
ACPA against vimentin	0.792 (0.457, 1.127)	<0.001	0.827 (0.490, 1.163)	<0.001
ACPA against histone	0.739 (0.410, 1.068)	<0.001	0.758 (0.426, 1.090)	<0.001
ACPA against alpha-enolase	0.790 (0.443, 1.136)	<0.001	0.840 (0.492, 1.187)	<0.001
IgA RF	0.525 (0.236, 0.814)	<0.001	0.536 (0.245, 0.827)	<0.001
IgG RF	0.163 (0.002, 0.325)	0.047	0.163 (0.001, 0.326)	0.049
IgM RF	0.454 (0.157, 0.750)	0.003	0.442 (0.146, 0.737)	0.004
Dependent Variable	**Anti-*F. nucleatum* coefficient (95% CI)**	**p-value**	**Anti-*F. nucleatum* coefficient (95% CI)**	**p-value**
Anti-CCP2	0.066 (-0.326, 0.459)	0.740	0.081 (-0.314, 0.476)	0.687
ACPA against vimentin	0.055 (-0.154, 0.264)	0.604	0.048 (-0.161, 0.256)	0.655
ACPA against histone	0.036 (-0.169, 0.241)	0.731	0.041 (-0.165, 0.247)	0.693
ACPA against alpha-enolase	0.179 (-0.034, 0.392)	0.100	0.181 (-0.032, 0.393)	0.095
IgA RF	-0.090 (-0.275, 0.094)	0.337	-0.081 (-0.266, 0.104)	0.388
IgG RF	0.080 (-0.020, 0.179)	0.117	0.084 (-0.016, 0.184)	0.098
IgM RF	-0.046 (-0.231, 0.138)	0.622	-0.024 (-0.206, 0.158)	0.799

ACPA = anti-citrullinated protein antibodies
All measures in this table were log base 2 transformed.
[a]Models were adjusted for age, sex, and smoking.

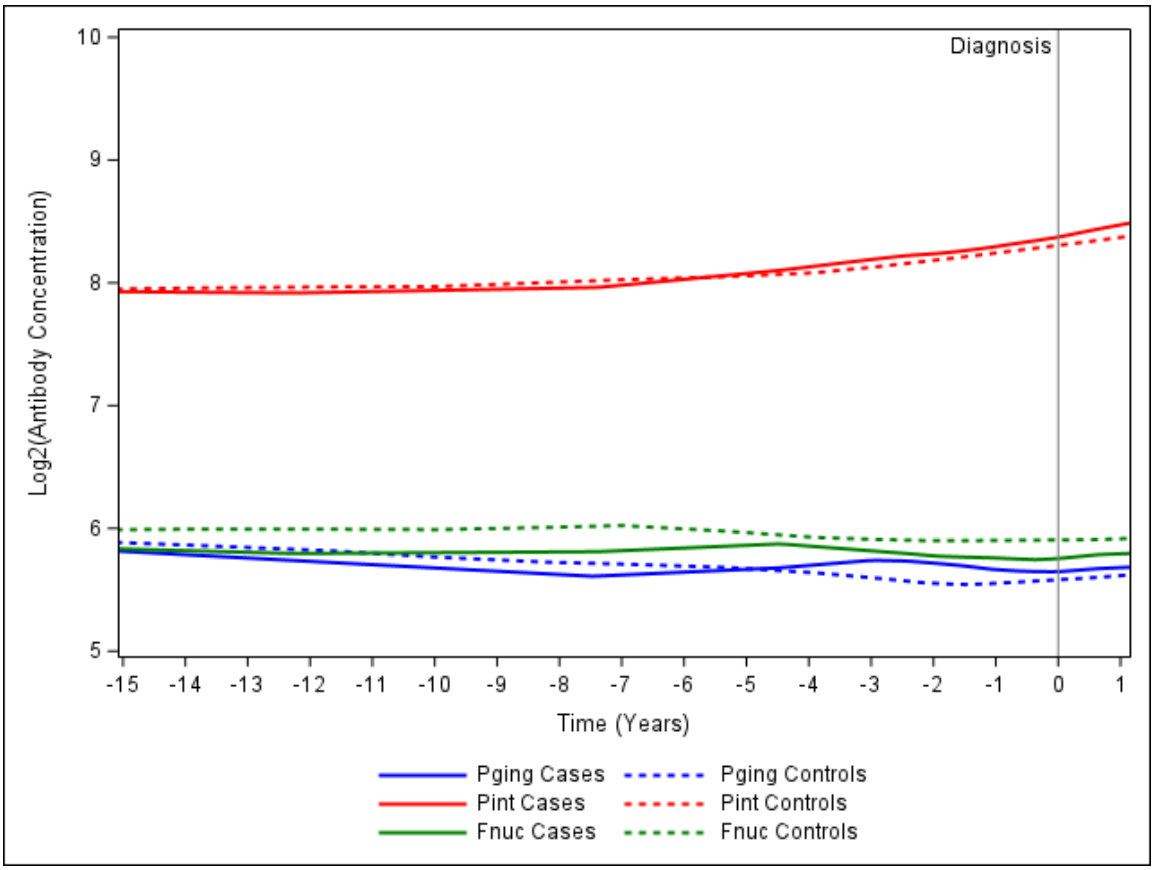


Figure 1: Pre-rheumatoid arthritis diagnosis concentrations of serum anti-bacterial antibodies (RA cases shown with solid lines, controls shown with dashed lines).

CHAPTER 4: ASSOCIATIONS BETWEEN PERIODONTITIS AND SERUM ANTI-MALONDIALDEHYDE-ACETALDEHYDE ANTIBODY CONCENTRATIONS IN RHEUMATOID ARTHRITIS: A CASE-CONTROL STUDY

Abstract

Background: Malondialdehyde–acetaldehyde (MAA) adducts lead to generation of anti-MAA autoantibodies and have been independently identified in inflamed periodontal and rheumatoid arthritis (RA) tissues. This study evaluates serum samples from RA cases and osteoarthritis (OA) controls to quantify associations between periodontal clinical measures, alveolar bone loss (ABL) and anti-*Porphyromonas gingivalis*, anti-*Prevotella intermedia*, and anti-*Fusobacterium nucleatum* antibody concentrations with anti-MAA antibody concentrations.

Methods: Participants (n=284 RA cases, n=330 OA controls) underwent periodontal clinical assessments and ABL measurements. Serum IgA, IgG and IgM anti-MAA and serum IgG anti-bacterial antibody concentrations were quantified by ELISA. Analyses utilized simple linear regression and multivariable adjusted models.

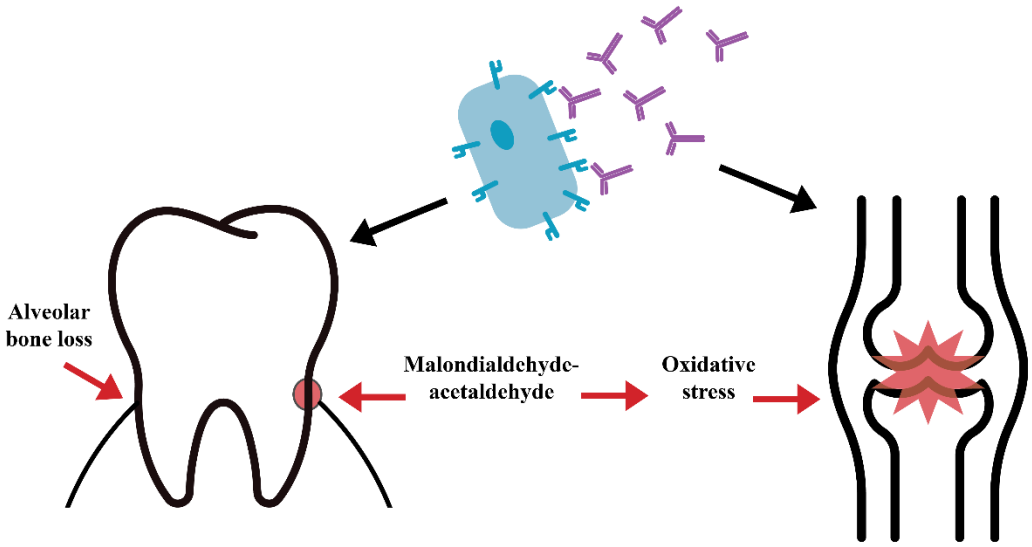
Results: No significant associations of periodontal clinical measures with serum anti-MAA were found. Moderate (p=0.038 and p=0.036, respectively) and high ABL (p=0.012 and p=0.014, respectively) in RA cases (but not in OA) were positively associated with IgG and IgM anti-MAA. Anti-*P. gingivalis* and anti-*P. intermedia* antibody concentrations were positively associated with IgA (p=0.001 for both), IgG (p=0.007 and p=0.034, respectively) and IgM anti-MAA antibody concentrations ($p<0.001$ and p=0.020, respectively) while anti-*F. nucleatum* was positively associated with IgG anti-MAA (p=0.042), findings that were similar across groups.

Conclusions: A positive association was demonstrated between ABL and serum IgG and IgM anti-MAA antibody concentrations that was unique to RA and not observed in OA. Serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations displayed

significant associations with anti-MAA antibody in both groups. These findings suggest MAA may play a role in the interrelationship between the periodontium and RA.

Key Words: rheumatoid arthritis, periodontitis, oxidative stress, *Porphyromonas gingivalis, Prevotella intermedia,* alveolar bone loss

Graphical Abstract



Highlights:

- No associations between periodontal clinical measures and serum anti-MAA were found
- Positive and statistically significant association noted between alveolar bone loss and serum IgG and IgM anti-MAA in RA cases
- No association between alveolar bone loss and serum anti-MAA in OA controls
- Serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* positively associated with serum anti-MAA
- MAA may play a role in the interrelationship between the periodontium and RA

Lee, J.A., Mikuls, T.R., Sayles, H.R., Thiele, G.M., Duryee, M.J., Payne, J.B. Associations between periodontitis and serum anti-malondialdehyde-acetaldehyde antibody concentrations in rheumatoid arthritis: A case-control study. J Periodontol. doi: 10.1002/JPER.23-0604

1 | Introduction

Periodontitis (PD) is a chronic oral disease (1). In addition to its impact on oral health, emerging evidence suggests that PD exerts systemic effects, contributing to the pathogenesis of various systemic inflammatory conditions (176), including rheumatoid arthritis (RA) (2).

RA is an autoimmune disease characterized by chronic inflammation of synovial joints frequently accompanied by bone erosion and joint deformity (177). The connection between PD and RA has been extensively investigated (178-180) as the two conditions share common inflammatory pathways (3), histopathologic features, and genetic/environmental risk factors (181). While studies have shown higher prevalence of PD in RA patients (102, 182) and improvements in RA disease activity following periodontal treatment (180, 183), further investigation is needed to fully understand the relationship between these inflammatory conditions.

Specific oral pathogens, such as *Porphyromonas gingivalis, Prevotella intermedia* and *Fusobacterium nucleatum*, have been postulated as a link between PD and RA (4-6). *P. gingivalis* is a keystone pathogen in PD and uniquely produces a peptidylarginine deiminase capable of citrullinating proteins; these citrullinated proteins can serve as autoantigens in susceptible patients, resulting in anti-citrullinated protein antibody (ACPA) production (7, 8). Studies have demonstrated an increased concentration of anti–*P. gingivalis* antibodies in RA patients and a positive correlation between anti–*P. gingivalis* antibody and circulating ACPA (9). *P. intermedia* has been shown to induce ACPA, albeit via a different mechanism than *P. gingivalis* (4) and *F. nucleatum* was more abundant in the salivary microbiota of early RA patients than healthy controls (5). Collectively, these findings suggest that the microbiota of the oral cavity may play a role in RA pathogenesis.

While the etiology of RA remains multifactorial and complex, oxidative stress, resulting from accumulation of reactive oxygen species, has also been implicated in its pathogenesis (15). Malondialdehyde-acetaldehyde (MAA) is a stable aldehyde compound formed through the interaction of malondialdehyde (MDA), which in contrast is a highly reactive aldehyde and a product of lipid peroxidation, with acetaldehyde (AA), another highly reactive metabolite of alcohol and other substances (16, 17). This immunogenic MAA adduct has been strongly linked to various inflammatory diseases, including RA and atherosclerosis (18, 184).

Anti-MAA antibody isotype responses are associated with ACPA in RA patients (18) and anti-MDA levels have been correlated with RA disease activity (70), though these antibodies recognize distinct epitopes. Our group reported that MAA-modified proteins were increased in the synovium of RA patients relative to osteoarthritis (OA) controls and co-localized with citrullinated proteins in inflamed synovial tissues, but not in OA tissues (18). In RA synovial tissues, MAA co-localized not only with citrulline but also mature B cells, which suggests MAA-adduct formation may have a role in local autoantibody production (19). In addition, inflamed human periodontal tissues were an extrasynovial source of MAA-modified proteins when compared to healthy gingival tissues, suggesting that inflammation of the periodontal tissues could lead to MAA generation and subsequent systemic inflammatory responses that have been demonstrated in RA (21). Further investigation is needed to determine the potential role of MAA in the relationship between PD and RA.

To better understand the significance of anti-MAA autoantibody concentrations on the association between PD and RA, serum samples from RA patients and OA controls from four U.S. Veterans Affairs Medical Centers and one academic medical center were evaluated (102). We sought to test the hypotheses that periodontal clinical measures, alveolar bone loss (ABL), and serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations are associated with anti-MAA serum autoantibody concentrations. We further sought to examine

whether associations of these measures with anti-MAA serum antibody differed between patients with RA and OA.

2 | Materials and Methods

2.1 | Study participants

Participants were recruited from October 20, 2010 to July 30, 2012 from four U.S. Veterans Affairs Medical Centers (Omaha, NE; Dallas, TX; Salt Lake City, UT; and Washington, DC) and a single academic coordinating center (University of Nebraska Medical Center, Omaha, NE) (102). From rheumatology, orthopedic, and primary care clinics, 617 participants were enrolled. RA patients fulfilled the 1987 American College of Rheumatology classification criteria (age of onset >18 years) (171), resulting in 287 RA cases. Three RA cases were missing data for anti-MAA antibody and thus excluded from this analysis (leaving 284 RA cases). Patients with OA (n = 330) served as controls based on the expectation that they were demographically similar to RA cases, as shown in a related pilot study (185). OA controls were selected after reviewing medical records, using either medical documentation or imaging results, demonstrating evidence of OA in the absence of inflammatory arthritis (102).

Inclusion criteria for the study were: 1) willingness/ability to provide informed consent; 2) age ≥19 years; 3) ≥9 posterior teeth (excluding third molars); and 4) RA or OA diagnosis after satisfying the aforementioned criteria. Patients were excluded due to: 1) tetracycline or related antibiotic use in the past 6 months; 2) antibiotic prophylaxis required prior to clinical assessment of periodontal parameters (e.g., recent total joint replacement or cardiac indication); 3) pregnant or breastfeeding; 4) history of cyclosporine or dilantin use; and 5) history of concomitant systemic inflammatory disease (e.g., systemic lupus erythematosus, ankylosing spondylitis).

2.2 | Ethical considerations

This study was approved by Institutional Review Boards at four U.S. Veterans Affairs Medical Centers (Omaha, NE; Dallas, TX; Salt Lake City, UT; and Washington, DC) and the

University of Nebraska Medical Center and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All study participants provided informed written consent prior to enrollment and informed consent allowed us to measure inflammatory mediators in banked blood.

2.3 | Sociodemographic and comorbidity assessments

Patient factors recorded at enrollment included: age, sex, race, body mass index (BMI, kg/m^2), and smoking status (current, former, or never). Self-reported comorbidities collected included: diabetes mellitus, hypertension, cardiovascular disease, and osteoporosis.

2.4 | Periodontal clinical measures

Periodontal clinical assessments were performed by a single dentist or periodontist at each site. Dental examiners were blinded to patient case status. Measurements included probing depths and gingival recession at 6 sites per tooth for all teeth (excluding third molars). Examiners were calibrated with a gold-standard periodontist (JBP) by satisfying 85% of probing depth and gingival recession measurements were within ±1 mm (102). PD was defined by the presence of clinical attachment loss of ≥6 mm on ≥2 teeth, and ≥1 site with probing depths of ≥5 mm (186). Bleeding on probing (BOP), number of missing teeth (out of 28 after excluding third molars), and presence of supragingival plaque, as a measurement of oral hygiene status, were recorded (102, 187).

2.5 | ABL measurements

A digital panoramic radiograph was obtained and imported into radiographic viewing software. Two trained examiners measured ≤24 sites per individual, both blinded to patient case status. Mesial sites on second premolars, first molars, and second molars and distal sites on first premolars, second premolars, and first molars were evaluated (172). The distance between the cemento-enamel junction (CEJ) and the alveolar crest and from the CEJ to the root apex were measured at each site. The radiographic examiners were calibrated by an oral and maxillofacial radiologist, also masked to participant case status. Interrater agreement was evaluated by having

both examiners reread a subset of radiographs (n = 69) and was strong (0.85, 95% CI: 0.77 to 0.91) (172). The mean percentage of ABL was determined with a CEJ-to-alveolar-crest distance >2 mm representing ABL (188). Mean percentage of bone loss, on a patient basis, was categorized into tertiles as follows for low (reference), moderate, and high ABL: ≤3.86%, >3.86% and ≤8.80%, and >8.80%, respectively, as previously described.(172) ABL data was missing in 11 RA and 12 OA participants (2 RA patients had missing panoramic radiographs and the remaining had restorations or overlapping of teeth that interfered with CEJ detection).

2.6 | Preparation of MAA Antigens

Soluble patient grade human serum albumin (HSA)* at 2 mg/mL was reacted with 2 mM MDA and 1 mM AA for 3 days at 37°C in 0.1M sodium phosphate dibasic buffer pH 7.2. Excess MDA and AA were dialyzed using 0.1M phosphate buffer. Presence of the 1,4 dihydropyridine MAA structure was demonstrated by fluorescence at an excitation of 398 nm and emission 460 nm. Further validation was done by ELISA using a rabbit anti-MAA antibody to determine reactivity to the antigen prior to using patient serum. HSA was used as the coating protein to focus our studies on antibody reactivity to MAA and antibody responses to HSA have consistently been shown to be negligible in RA.

2.7 | Anti-MAA assays

Anti-MAA antibodies (IgA, IgG, and IgM; ng/ml) were quantified using ELISA (18, 19, 73). This method consisted of coating 2 µg/well HSA and MAA-modified HSA (HSA-MAA) on ELISA plates using bicarbonate buffer pH 9.6 and incubated overnight at 4°C. Plates were then washed with PBS-T pH 7.2, blocked using 2% casein buffer pH 7.2, and serum was incubated at a 1:1000 dilution in 0.25% casein PBS-T pH 7.2. Serum antibodies were detected using horse radish peroxidase (HRP) goat anti-human IgA, IgG, or IgM secondary antibody.† Plates were developed using tetramethylbenzidine (TMB) substrate and absorbance determined at 450 nm

using an Epoch microplate reader with Gen5 Software.[‡] A standard curve was generated onto each plate using 50 ng/mL of purified human IgA, IgG, or IgM.[†] Briefly, the isotypes were diluted in bicarbonate buffer (50 ng/mL) and then serially diluted down the plate. Antibody concentrations were determined by extrapolation from these standard antibody curves. Subsequently, serum samples were evaluated for reactivity and anti-MAA antibody concentrations were determined by subtracting HSA reactivity from HSA-MAA reactivity. Patient samples were run in duplicate with the coefficient of variation (CV) ranging between 1 to 10%. Samples over a 10% CV were retested.

2.8 | Bacterial serologies

Serum concentrations of IgG antibodies to several periodontal pathogens were measured using ELISA. Outer membrane antigen (OMA) to *P. gingivalis* strain 381, *P. intermedia* strain VPI 4197, and *F. nucleatum* strain VPI 4355 (American Type Culture Collection, ATCC)[§] were purified. Broth cultures were centrifuged at 6500 rpm for 20 minutes, pellets re-suspended in 0.15M sodium chloride with protease inhibitors, and frozen overnight at -80°C. This freeze-thaw method was done twice, followed by sonication on ice, and centrifuged at 12,000 x g for 20 minutes. Supernatants were collected and centrifuged at 100,000 x g for 1 hour at 4°C. Protein determination was done using the BCA method. OMA at 2 µg/well in bicarbonate buffer was coated onto ELISA plates overnight at 4°C. Plates were washed with PBS-T pH 7.2, blocked using 2% casein buffer pH 7.2, and patient serum incubated at a 1:10,000 dilution in 0.25% casein PBS-T pH 7.2. Serum antibodies were detected using an HRP Goat anti-human IgG secondary antibody.[†] Plates were developed using TMB substrate and absorbance determined at 450 nm using an Epoch microplate reader with Gen5 Software.[‡] A standard curve was made using known concentrations of purified human IgG as detailed above.[†] Bacterial antibody concentrations (µg/ml) were determined from a standard curve, and then log-transformed for

analysis (102). Samples were run in duplicate with the CV ranging between 1 to 10%. Samples over a 10% CV were retested.

2.9 | Statistical analysis

Participant characteristics were compared between RA cases and OA control groups using t-tests for continuous measures and chi-square tests for categorical measures. Lower limit of detection values were imputed for autoantibody and bacterial antibody measures which fell below that point and all such measures were log (base 2) transformed.

Primary analyses investigated associations between clinical periodontal measures, including the protocol definition of PD (186), the number of missing teeth, and the percentages of sites with plaque, BOP, probing depths ≥5 mm, and attachment loss ≥5 mm and anti-MAA antibodies. Initially, simple linear regression models examined associations between each of these clinical measures (independent variables) and each anti-MAA antibody isotype (dependent variables). Possible interactions were evaluated by adding RA versus OA status and an interaction term for RA/OA status by the initial independent variable. Finally, multivariable models were built with covariates including age, sex, race, smoking status, and RA versus OA status, which were chosen because they showed significance in a previous paper from our group in multivariable models for at least one anti-MAA isotype (189), and diabetes, which is a risk factor for PD (190). When interaction terms showed evidence of statistical interaction (conservative threshold defined as a $p<0.10$), multivariable analyses were run separately for RA and OA participants. When there was no evidence of an interaction ($p\geq0.10$), the multivariable models simply included an indicator variable for RA versus OA status as a covariate.

Secondary analyses examined associations between ABL, anti-*P. gingivalis*, anti-*P. intermedia,* and anti-*F. nucleatum,* and anti-MAA antibody isotypes. ABL was categorized into

low (≤3.86%; reference), moderate (>3.86% and ≤8.80%), and high (>8.80%) based on tertiles of mean ABL values (172). A similar approach as above was used beginning with simple linear regressions, then evaluating interactions, and finally using multivariable models with covariates and the approach for either stratifying the analysis or just adding an RA/OA status indicator. All analyses were performed using Stata v17.0.‖

3 | Results

3.1 | Participant characteristics

Patient characteristics are shown in Table 1. RA cases did not differ from OA controls with respect to age, sex, and race. RA cases were more likely to be current (19% vs. 11%) or former smokers (43% vs. 35%) than OA controls (p<0.001). In contrast, OA controls had higher mean BMI (p=0.001), and greater prevalence of self-reported diabetes mellitus (p=0.048) and hypertension (p=0.004) than participants with RA. There were no differences between RA cases and OA controls regarding cardiovascular disease and osteoporosis frequency. RA cases were more likely to be diagnosed with PD than OA controls (p=0.021). No differences were noted between RA cases and OA controls with respect to other periodontal measures. Mean serum IgA (p=0.004), IgG (p=0.015), and IgM (p=0.001) anti-MAA antibody concentrations were universally higher in individuals with RA than those with OA (Table 1).

3.2 | Interaction Terms of RA versus OA status

Evidence of statistical interaction was noted between the following independent variables and serum anti-MAA antibody isotypes with respect to RA versus OA status: missing teeth for serum IgA anti-MAA antibody isotype and ABL for all three anti-MAA autoantibody isotypes (Supplementary Table 1). Therefore, for these independent variables only, multivariable analyses were done separately by RA versus OA status (Table 2, Figure 1).

3.3 | Periodontal measures and anti-MAA antibody isotype concentrations

In simple linear regression models, the percentage of sites with plaque was significantly positively associated with serum IgA anti-MAA antibody concentrations (p=0.039) while the percentage of sites with BOP was negatively associated with IgG anti-MAA (p=0.017). PD, missing teeth, and percentage of sites with probing depths ≥5 mm and attachment loss ≥5 mm were not associated with serum anti-MAA antibody isotype concentrations in simple models (Supplementary Table 2).

In multivariable adjusted models, no significant associations were noted between missing teeth and serum IgA anti-MAA antibody for either RA or OA groups. Likewise, no significant associations were found between the remaining periodontal clinical measures and serum anti-MAA antibody concentrations for the overall participant population (Table 2).

3.4 | ABL and anti-MAA antibody isotype concentrations

Given evidence of interaction between RA-OA case status and ABL, models were examined separately for RA cases and OA controls. Using the simple linear regression model, moderate (p=0.024) and high ABL (p=0.031), versus low ABL, were significantly positively associated with serum IgG anti-MAA in RA cases. In contrast, high (p=0.005) ABL in OA controls, versus low ABL, was significantly negatively associated with serum IgM anti-MAA (Supplementary Table 3). In the multivariable adjusted models, no significant associations were observed between ABL and serum IgA anti-MAA antibody concentrations in either RA or OA groups (Figure 1A). However, moderate (p=0.038 and p=0.036, respectively) and high ABL (p=0.012 and p=0.014, respectively), versus low ABL, were significantly and positively associated with serum IgG and IgM anti-MAA in RA cases with non-significant negative associations in OA comparators (Figure 1B and C).

3.5 | Anti-bacterial antibody concentrations and anti-MAA antibody isotype concentrations

In a simple linear regression model, anti-*P. gingivalis* and anti-*P. intermedia* serum antibody concentrations were significantly associated with serum IgA ($p<0.001$ and $p=0.001$, respectively), IgG ($p<0.001$ for both), and IgM anti-MAA antibody concentrations ($p=0.001$ and $p=0.035$, respectively) while anti-*F. nucleatum* was associated only with IgG anti-MAA ($p=0.001$) (Supplementary Table 4). In multivariable adjusted models, anti-*P. gingivalis* and anti-*P. intermedia* serum antibody concentrations were still significantly associated with serum IgA ($p=0.001$ for both), IgG ($p=0.007$ and $p=0.034$, respectively) and IgM anti-MAA antibody concentrations ($p<0.001$ and $p=0.020$, respectively), while anti-*F. nucleatum* was still associated only with IgG anti-MAA ($p=0.042$) (Table 3).

4 | Discussion

In this study, PD and periodontal clinical measures were not associated with serum anti-MAA antibody concentrations; however, we observed a positive and statistically significant association between ABL and serum IgG and IgM anti-MAA antibody concentrations in RA cases, a finding that differed significantly from those with OA. This finding aligns with a recent paper demonstrating that anti-MDA antibodies, which share substantial overlap with anti-MAA antibody, induce osteoclast differentiation and bone erosion in RA, highlighting the current relevance of our findings (72). We also found that serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations were significantly positively associated with serum anti-MAA antibody concentrations in the entire study population.

The presence of MAA in various inflammatory diseases has garnered considerable interest over the past decade. Direct MAA-adducted protein exposure in lungs of mice, intended to replicate the combined effects of alcohol and smoke exposure, leads to increased airway

inflammation, characterized by neutrophil infiltration and chemokine release (191). Human studies have further supported involvement of MAA in inflammatory processes, revealing increased tissue expression of MAA-adducts in lung cells and increased serum IgA anti-MAA antibodies in individuals with alcohol use disorders who also smoked compared to healthy, non-smoker individuals (192). Chronic inflammation induced by MAA adduct formation has also been implicated in atherosclerosis, an effect that appears to be mediated through generation of lipid-laden endothelial cells and macrophages (184). The presence of MAA adducts was found in tissues of atheromatous lesions and IgM, IgG and IgA isotypes of anti-MAA were differentially and significantly associated with non-obstructive coronary artery disease, acute myocardial infarction, or obstructive multi-vessel coronary artery disease (193). In RA patients, serum IgA anti-MAA antibody concentrations were associated with increased coronary artery calcium and modified the relationship between American College of Cardiology/American Heart Association 10-year risk score and coronary artery calcium (194). These studies underscore the significance of MAA as a potential biomarker of inflammation and its role in various systemic inflammatory diseases associated with oxidative stress.

Our group previously demonstrated that MAA adduct formation is increased in RA synovial tissue compared to OA synovial tissue, anti-MAA antibody isotype responses are strongly associated with ACPA, and MAA adducts co-localize with citrullinated proteins in inflamed synovial tissues in RA (18). Moreover, concentrations of IgG and IgM anti-MAA antibodies were approximately three-fold higher in RA synovial fluid compared to paired serum in these patients (19). This highlights the potential role of MAA in local inflammatory processes within the RA joint. Using MAA-modified forms of an alternative molecule as the plating antigen, low-density lipoprotein (MAA-LDL) rather than albumin as used in our study, others showed that concentrations of IgA antibody to MAA-LDL correlated with antibodies to the periodontal pathogens *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* in addition to

RA-associated autoantibodies (195). Serum IgA and IgM anti-MAA antibodies were found to be associated with interstitial lung disease among RA patients (196), suggesting a potential role of MAA-related immune responses in the pathogenesis of RA-interstitial lung disease. Moreover, elevation of IgG and IgA anti-MAA autoantibodies prior to RA diagnosis and their appearance later in the preclinical course than ACPA and rheumatoid factor (RF) suggests involvement of MAA in the transition from subclinical autoimmunity to the clinical presentation of RA (73). Altogether, these findings emphasize the significance of MAA in the pathogenesis of RA and its potential as a biomarker and therapeutic target for RA management.

Limited data exist on the relationship between PD and MAA formation. Our group's previous study reported simultaneous detection of citrullinated, carbamylated, and MAA adduct modified proteins in inflamed periodontal tissues, suggesting that the periodontium may serve as an extrasynovial source of these proteins (21). While our current study showed no association between several periodontal clinical measures and anti-MAA antibody using modified human albumin as the coating protein, it is worth noting that Akhi et al. reported salivary IgA antibodies to MAA-LDL were associated with 4–5 mm probing depths in patients with chronic coronary artery disease. The authors concluded that the increased humoral immune response to oxidized epitopes such as MAA-LDL could partly explain the relationship between PD and systemic disease (93).

To our knowledge, no prior studies have investigated the direct relationship between ABL and anti-MAA antibody, making our findings novel and noteworthy. Our study demonstrated moderate and high ABL in RA cases were positively associated with serum IgG and IgM anti-MAA. These results align with the study by Gonzalez et al., which found that greater ABL was associated with higher ACPA levels (172), supporting the concept that ABL may be related to RA and could possibly be considered an extra-articular manifestation of the condition (Figure 2). Notably, the relationship of ABL with anti-MAA antibody differed

markedly in RA cases compared to OA controls. In addition to suggesting that ABL may be a manifestation of RA (i.e., with the alveolar bone serving as another "joint" with bone destruction), these findings also suggest that anti-MAA antibody may conspire with other cofactors that are characteristic of RA and absent in OA (e.g., systemic inflammation) in driving ABL.

The association between anti-bacterial antibody concentrations and anti-MAA antibody isotype concentrations in our study adds to the growing body of evidence regarding the role of specific oral pathogens in RA pathogenesis. Our group recently reported in RA cases that serum anti-*P. intermedia* was significantly and positively associated with anti-CCP2 (a global ACPA measure), ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA, IgG, and IgM RF autoantibody concentrations prior to RA diagnosis, while anti-*P. gingivalis* and anti-*F. nucleatum* were not associated with RA autoantibody concentrations (197). Akhi et al. explored the association of salivary IgA to MAA-LDL and periodontal pathogens (92, 93, 198). Saliva IgA and IgG antibodies binding to MAA-LDL and copper oxidized-LDL were found in all patient samples and were associated with the salivary levels of IgA and IgG to *P. gingivalis* and *A. actinomycetemcomitans*. Additionally, saliva IgA antibodies to MAA-LDL cross-reacted specifically with *P. gingivalis*, suggesting that these antibodies may participate in immune reactions involved in LDL oxidation through molecular mimicry (92). Salivary IgA antibody to MAA-LDL also showed cross-reactivity with *A. actinomycetemcomitans* (198). The positive associations between these anti-bacterial antibody concentrations and anti-MAA antibodies and the possible existence of cross-reactive epitopes in oxidized proteins and periodontal bacteria suggest that specific oral pathogens implicated in PD might play a role in the mucosal immune response linking RA and PD.

This study has limitations. Though valuable for identifying associations, the study's case-control design does not establish causality. Additionally, study participants were comprised of a

relatively high proportion of men to women (63% versus 37%, respectively), which might limit the generalizability of the results to a more typical RA population (175). The reliance on self-reported comorbidities introduces the possibility of recall bias, potentially influencing accuracy and completeness of the data. The anti-MAA assay used in this study (and others from our group) used modified HSA. It is possible that incorporating more RA-relevant antigens (e.g., MAA-modified vimentin, fibrinogen, or type II collagen) could result in different assay performance. Despite these limitations, this study contributes valuable insights into the potential associations between MAA, RA, periodontal pathogens, and ABL, and provides a foundation for further research in this field.

5 | Conclusion

In conclusion, we noted a significant positive association between ABL and serum IgG and IgM anti-MAA antibody concentrations in RA cases, an association unique from that observed in OA control participants. Serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations also were positively associated with serum anti-MAA antibody concentrations, indicating a possible relationship between oral pathogens and generation of anti-MAA antibodies. This study suggests a potential role of MAA-adduct formation and anti-MAA immune responses in the relationship between the periodontium and RA. Longitudinal studies are warranted to further confirm and explore these associations.

FOOTNOTES

*Talecris Biotherapeutics, Inc, Research Triangle Park, NC

†Jackson ImmunoResearch, West Grove, PA

‡Agilent Technologies, Santa Clara, CA

§*P. gingivalis* strain 381*, P. intermedia* strain VPI 4197*,* and *F. nucleatum* strain VPI 4355 American Type Culture Collection, Bethesda, MD

[‖] Stata v17.0, StataCorp, College Station, TX

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CONFLICT OF INTEREST

TRM has served as a consultant for Horizon Therapeutics (Deerfield, IL), Pfizer (New York, NY), Sanofi (Bridgewater, NJ), and UCB (Atlanta, GA) and received research support from Horizon Therapeutics (Deerfield, IL). None of the other authors has a conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

Table 1 – Participant characteristics

Characteristics	**Total** (n = 614)	**Rheumatoid arthritis cases** (n = 284)	**Osteoarthritis controls** (n = 330)	***p*-value**
Sociodemographics				
Age (years), mean (SD)	59.2 (11.2)	58.9 (11.8)	59.5 (10.5)	0.539*
Sex, n (%)				
Female	236 (38)	104 (37)	132 (40)	0.391†
Male	378 (62)	180 (63)	198 (60)	
Race, n (%)				
White	451 (73)	212 (75)	239 (72)	0.224†
African American	119 (19)	48 (17)	71 (22)	
Other‡	44 (7)	24 (8)	20 (6)	
Health-Related Measures and Comorbidities				
Body mass index (kg/m^2), mean (SD)	30.9 (6.8)	29.9 (6.7)	31.7 (6.8)	0.001*
Smoking status, n (%)				
Never	285 (46)	108 (38)	177 (54)	<0.001†
Former	239 (39)	122 (43)	117 (35)	
Current	90 (15)	54 (19)	36 (11)	
Diabetes mellitus, n (%)	132 (22)	51 (18)	81 (25)	0.048†
Hypertension, n (%)	317 (52)	129 (45)	188 (57)	0.004†
Cardiovascular disease, n (%)	69 (11)	36 (13)	33 (10)	0.295†
Osteoporosis, n (%)	81 (13)	31 (11)	50 (15)	0.122†
Periodontal Characteristics				
Periodontitis§, n (%)	186 (30)	99 (35)	87 (26)	0.021†
Missing teeth, mean (SD)	3.28 (3.31)	3.20 (3.08)	3.35 (3.50)	0.601*
Percentage of sites with plaque‖, n (%)				0.310†
High	307 (50)	148 (52)	159 (48)	
Low	306 (50)	135 (48)	171 (52)	
Percentage of sites with bleeding on probing‖, n (%)				0.955†
High	309 (50)	143 (51)	166 (50)	
Low	304 (50)	140 (49)	164 (50)	
Percentage of sites with probing depths ≥5 mm‖, n (%)				0.193†
High	301 (49)	147 (52)	154 (47)	
Low	312 (51)	136 (48)	176 (53)	

Percentage of sites with attachment loss ≥5 mm‖, n (%)				0.770†
High	308 (50)	144 (51)	164 (50)	
Low	305 (50)	139 (49)	166 (50)	
Alveolar bone loss tertiles¶, n (%)				0.183†
Low	195 (33)	85 (31)	110 (35)	
Moderate	198 (34)	86 (32)	112 (35)	
High	198 (34)	102 (37)	96 (30)	
Serum Measures				
IgA Anti-MAA, mean (SD)	7.31 (1.88)	7.54 (1.65)	7.11 (2.04)	0.004*
IgG Anti-MAA, mean (SD)	8.40 (1.29)	8.54 (1.24)	8.29 (1.33)	0.015*
IgM Anti-MAA, mean (SD)	8.84 (1.75)	9.09 (1.71)	8.62 (1.76)	0.001*

Abbreviations: MAA, malondialdehyde-acetaldehyde.

*Student's t-test, †Pearson chi-square test.

‡This category is composed of individuals who identified as the following: American Indian or Alaska Native (n = 7), Asian (n = 4), Native Hawaiian or Other Pacific Islander (n = 5), Hispanic, Black or African-American (n = 4), Hispanic, White (n = 8), Hispanic, Other Race -"Hispanic" (n = 11), Hispanic, Other Race - "Latina" (n = 1), and Hispanic, Other Race - "Spanish" (n = 2), Other race "African" (n = 1), and Other Race "Mother is Arab and Sicilian" (n = 1).

§Periodontitis was defined *a priori* according to the definition of Machtei et al.(186) as the presence of clinical attachment loss ≥6 mm on ≥2 teeth and one or more sites with probing depths ≥ 5 mm.

‖Median split categories of 50% high and 50% low were used.

¶Mean (patient basis) percentage bone loss tertiles are as follows for low, moderate, and high ABL: ≤3.86%, >3.86% and ≤8.80%, and >8.80%, respectively.

Table 2 – Multivariable adjusted* associations between periodontal measures and serum anti-malondialdehyde-acetaldehyde (MAA) antibody isotype concentrations

Periodontal Characteristics	**IgA Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgG Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgM Anti-MAA β-coefficient (95% CI)**	***p*-value**
Periodontitis[†]	0.204 (-0.146, 0.553)	0.253	0.019 (-0.218, 0.255)	0.876	0.275 (-0.050, 0.600)	0.097
Missing teeth (0 to 28)	RA: -0.009 (-0.074, 0.056) OA: -0.067 (-0.136, 0.000)	0.786 0.051	0.017 (-0.016, 0.049)	0.311	0.012 (-0.032, 0.057)	0.578
Percentage of sites with plaque (High versus Low)[‡]	0.264 (-0.037, 0.566)	0.086	0.039 (-0.166, 0.243)	0.710	-0.059 (-0.340, 0.222)	0.680
Percentage of sites with bleeding on probing (High versus Low)[‡]	0.236 (-0.101, 0.573)	0.170	-0.065 (-0.293, 0.163)	0.575	0.175 (-0.138, 0.489)	0.273
Percentage of sites with probing depths ≥5 mm (High versus Low)[‡]	0.081 (-0.228, 0.391)	0.606	-0.051 (-0.261, 0.158)	0.629	0.066 (-0.222, 0.354)	0.652
Percentage of sites with attachment loss ≥5 mm (High versus Low)[‡]	0.141 (-0.190, 0.471)	0.404	0.066 (-0.157, 0.290)	0.561	0.008 (-0.300, 0.316)	0.961

Abbreviations: MAA, malondialdehyde-acetaldehyde.

*Models were adjusted for age, sex, race, smoking status, diabetes, and rheumatoid arthritis (RA) versus osteoarthritis (OA) status; associations of IgA anti-MAA with missing teeth stratified by RA versus OA disease status due to evidence of interaction.

[†]Periodontitis was defined *a priori* according to the definition of Machtei et al.(186) as the presence of clinical attachment loss ≥6 mm on ≥2 teeth and one or more sites with probing depths ≥5 mm.

[‡]Median split categories of 50% high and 50% low were used.

Table 3 –Multivariable adjusted* associations between serum anti-bacterial antibody concentrations and serum anti-malondialdehyde-acetaldehyde (MAA) antibody isotype concentrations

	Multivariable Adjusted Models					
Anti-bacterial antibody concentrations	**IgA Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgG Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgM Anti-MAA β-coefficient (95% CI)**	***p*-value**
Anti-*P. gingivalis*, ug/mL	0.195 (0.085, 0.306)	0.001	0.104 (0.029, 0.180)	0.007	0.204 (0.101, 0.307)	<0.001
Anti-*P. intermedia*, ug/mL	0.305 (0.127, 0.484)	0.001	0.131 (0.010, 0.253)	0.034	0.199 (0.032, 0.366)	0.020
Anti-*F. nucleatum*, ug/mL	0.050 (-0.066, 0.166)	0.399	0.081 (0.003, 0.160)	0.042	0.095 (-0.013, 0.203)	0.086

Abbreviations: MAA, malondialdehyde-acetaldehyde; RA, rheumatoid arthritis; OA, osteoarthritis.

Serum anti-bacterial antibody concentrations in this table were log base 2 transformed.

*****Models were adjusted for age, sex, race, smoking status, diabetes, and rheumatoid arthritis versus osteoarthritis status.

Figure 1A

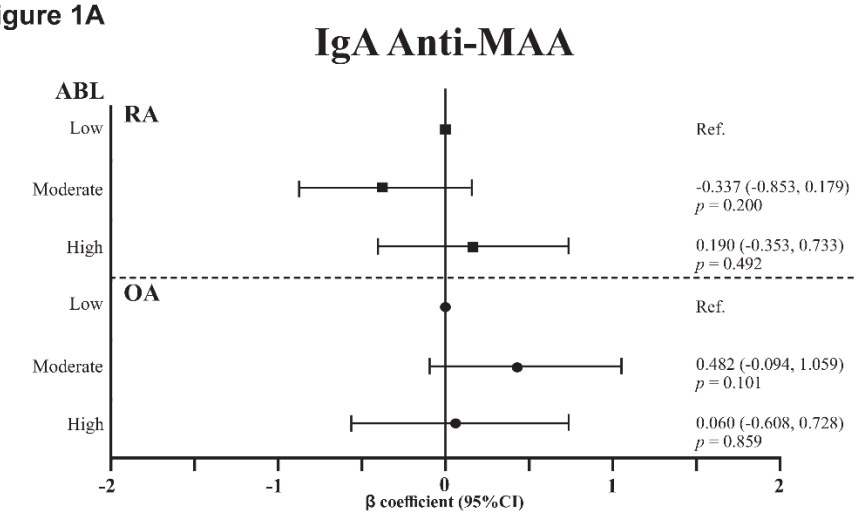


Figure 1B

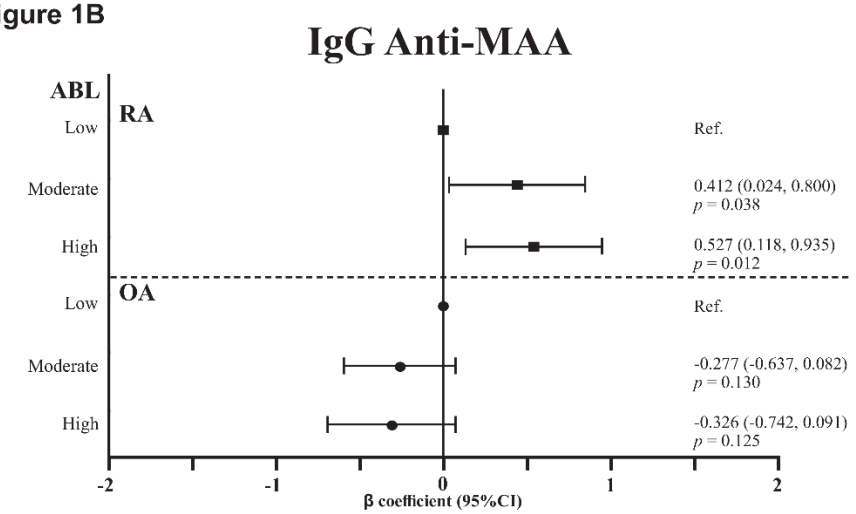


Figure 1C

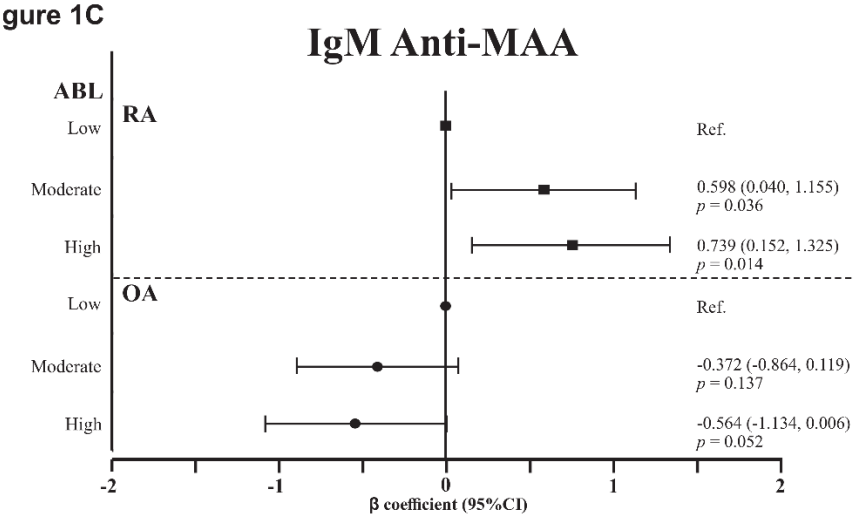


Figure 1: Forest plots showing results of multivariable adjusted associations between alveolar bone loss (ABL) and serum IgA (Figure 1A), IgG (Figure 1B), and IgM (Figure 1C) anti-malondialdehyde-acetaldehyde (MAA) antibody isotype concentration by rheumatoid arthritis (RA) versus osteoarthritis (OA) status. Mean (patient basis) percentage ABL tertiles are as follows for low, moderate, and high ABL: ≤3.86%, >3.86% and ≤8.80%, and >8.80%, respectively. Models were adjusted for age, sex, race, smoking status, and diabetes. Overall *p*-values using omnibus tests; for RA: IgA $p = 0.098$; IgG $p = 0.031$; IgM $p = 0.034$; for OA: IgA $p = 0.178$; IgG $p = 0.223$; IgM $p = 0.134$. Abbreviations: MAA, malondialdehyde-acetaldehyde; ABL, alveolar bone loss; RA, rheumatoid arthritis; OA, osteoarthritis.

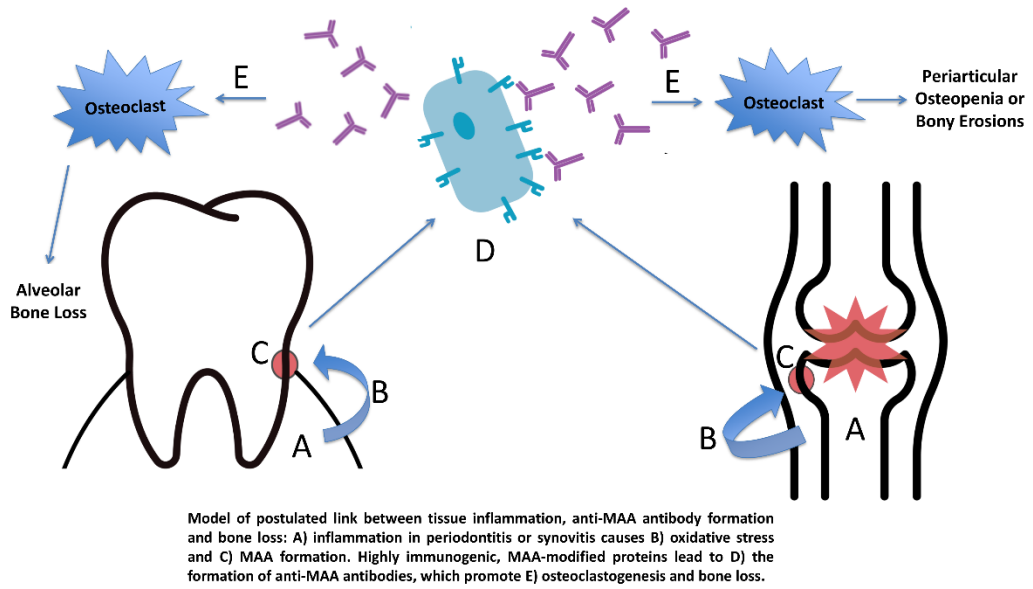


Figure 2: Schema showing postulated link between tissue inflammation, anti-malondialdehyde-acetaldehyde anti-MAA antibody formation and bone loss in rheumatoid arthritis and periodontitis. Abbreviation: MAA, malondialdehyde-acetaldehyde.

Supplementary Table 1 – *P*-values for the interaction terms of rheumatoid arthritis versus osteoarthritis status and periodontal measures, alveolar bone loss, and serum anti-bacterial antibody concentrations when predicting serum anti-malondialdehyde-acetaldehyde (MAA) antibody isotype concentrations

Periodontal Characteristics	**IgA Anti-MAA *p*-value**	**IgG Anti-MAA *p*-value**	**IgM Anti-MAA *p*-value**
Periodontitis*	0.454	0.651	0.668
Missing teeth (0 to 28)	0.050	0.742	0.106
Percentage of sites with plaque (High versus Low)†	0.749	0.267	0.142
Percentage of sites with bleeding on probing (High versus Low)†	0.761	0.780	0.148
Percentage of sites with probing depths ≥5mm (High versus Low)†	0.635	0.940	0.294
Percentage of sites with attachment loss ≥5 mm (High versus Low)†	0.838	0.203	0.361
Alveolar bone loss (tertiles)	0.010	0.068	0.030

Anti-bacterial antibody concentrations	**IgA Anti-MAA *p*-value**	**IgG Anti-MAA *p*-value**	**IgM Anti-MAA *p*-value**
Anti-*P. gingivalis*, ug/mL	0.787	0.625	0.340
Anti-*P. intermedia*, ug/mL	0.689	0.717	0.957
Anti-*F. nucleatum*, ug/mL	0.311	0.990	0.616

Abbreviation: MAA, malondialdehyde-acetaldehyde.
*Periodontitis was defined *a priori* according to the definition of Machtei et al.[29] as the presence of clinical attachment loss ≥6 mm on ≥2 teeth and one or more sites with probing depths ≥5 mm.
†Median split categories of 50% high and 50% low were used.

Supplementary Table 2 – Simple linear regression models of associations between periodontal measures and serum anti-malondialdehyde-acetaldehyde (MAA) antibody isotype concentrations

Periodontal Characteristics	**IgA Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgG Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgM Anti-MAA β-coefficient (95% CI)**	***p*-value**
Periodontitis*	0.213 (-0.112, 0.538)	0.199	0.074 (-0.149, 0.297)	0.512	0.079 (-0.224, 0.381)	0.610
Missing teeth (0 to 28)	-0.038 (-0.083, 0.007)	0.096	0.018 (-0.013, 0.049)	0.256	-0.011 (-0.053, 0.031)	0.607
Percentage of sites with plaque (High versus Low)†	0.315 (0.016, 0.613)	0.039	-0.057 (-0.262, 0.148)	0.588	-0.063 (-0.341, 0.216)	0.658
Percentage of sites with bleeding on probing (High versus Low)†	0.272 (-0.026, 0.570)	0.074	-0.249 (-0.453, -0.045)	0.017	0.214 (-0.064, 0.492)	0.130
Percentage of sites with probing depths ≥5 mm (High versus Low)†	0.133 (-0.166, 0.432)	0.382	-0.018 (-0.223, 0.187)	0.864	-0.063 (-0.341, 0.215)	0.657
Percentage of sites with attachment loss ≥5 mm (High versus Low)†	0.085 (-0.214, 0.384)	0.578	0.125 (-0.079, 0.330)	0.229	-0.179 (-0.457, 0.099)	0.207

Abbreviations: MAA, malondialdehyde-acetaldehyde.

*Periodontitis was defined *a priori* according to the definition of Machtei et al.[29] as the presence of clinical attachment loss ≥6 mm on ≥2 teeth and one or more sites with probing depths ≥5 mm.

†Median split categories of 50% high and 50% low were used.

Supplementary Table 3 – Simple linear regression models of associations between alveolar bone loss (ABL) and serum anti-malondialdehyde-acetaldehyde (MAA) antibody isotype concentrations by rheumatoid arthritis (RA) versus osteoarthritis (OA) status

RA Only

Alveolar bone loss tertiles*	**IgA Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgG Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgM Anti-MAA β-coefficient (95% CI)**	***p*-value**
Low ABL	Ref.	----†	Ref.	----†	Ref.	----†
Moderate ABL	-0.324 (-0.824, 0.176)	0.203	0.429 (0.056, 0.803)	0.024	0.305 (-0.218, 0.829)	0.252
High ABL	0.319 (0.161, 0.800)	0.192	0.396 (0.037, 0.754)	0.031	0.188 (-0.315, 0.690)	0.463

OA Only

Alveolar bone loss tertiles*	**IgA Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgG Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgM Anti-MAA β-coefficient (95% CI)**	***p*-value**
Low ABL	Ref.	----†	Ref.	----†	Ref.	----†
Moderate ABL	0.389 (-0.154, 0.933)	0.160	-0.136 (-0.486, 0.214)	0.446	-0.431 (-0.897, 0.036)	0.070
High ABL	-0.121 (-0.687, 0.445)	0.674	-0.084 (-0.449, 0.280)	0.647	-0.695 (-1.180, -0.209)	0.005

Abbreviations: MAA, malondialdehyde-acetaldehyde; ABL, alveolar bone loss; RA, rheumatoid arthritis; OA, osteoarthritis.

*Mean (patient basis) percentage alveolar bone loss (ABL) tertiles are as follows for low, moderate, and high ABL: ≤3.86%, >3.86% and ≤8.80%, and >8.80%, respectively.

†Overall *p*-values using omnibus tests; for RA: IgA $p = 0.031$; IgG $p = 0.041$; IgM $p = 0.512$; for OA: IgA $p = 0.169$; IgG $p = 0.744$; IgM $p = 0.018$

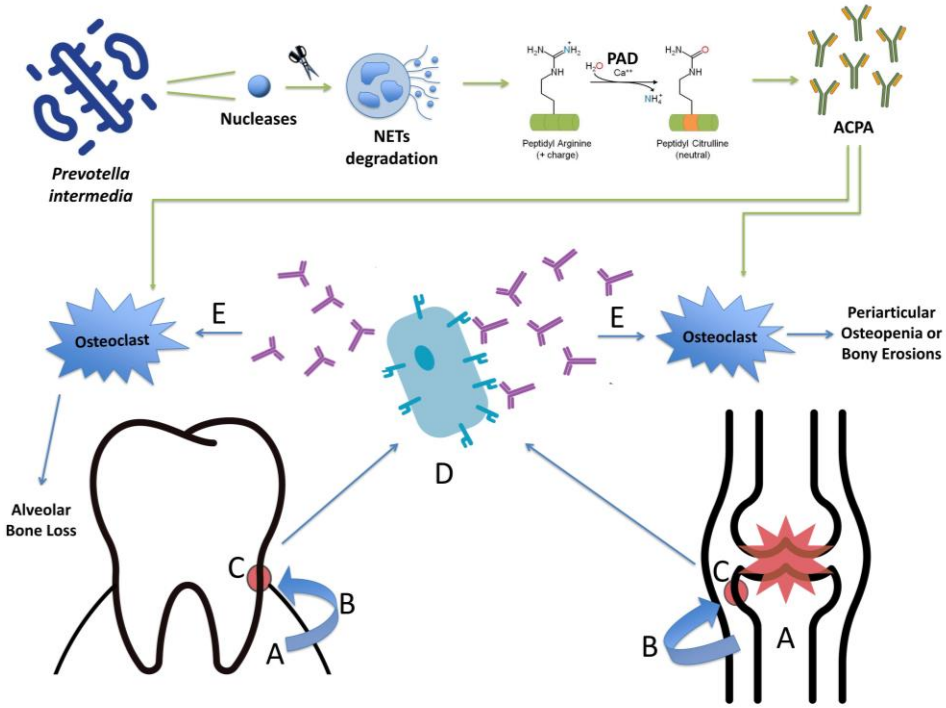
Supplementary Table 4 – Simple linear regression models of associations between serum anti-bacterial antibody concentrations and serum anti-malondialdehyde-acetaldehyde (MAA) antibody isotype concentrations

	Simple Linear Regression Models					
Anti-bacterial antibody concentrations	**IgA Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgG Anti-MAA β-coefficient (95% CI)**	***p*-value**	**IgM Anti-MAA β-coefficient (95% CI)**	***p*-value**
Anti-*P. gingivalis*, ug/mL	0.204 (0.096, 0.312)	<0.001	0.158 (0.084, 0.232)	<0.001	0.172 (0.072, 0.273)	0.001
Anti-*P. intermedia*, ug/mL	0.306 (0.133, 0.478)	0.001	0.229 (0.111, 0.347)	<0.001	0.174 (0.013, 0.336)	0.035
Anti-*F. nucleatum*, ug/mL	0.098 (-0.017, 0.213)	0.096	0.133 (0.055, 0.211)	0.001	0.105 (-0.002, 0.212)	0.054

Abbreviation: MAA, malondialdehyde-acetaldehyde.
Serum anti-bacterial antibody concentrations in this table were log base 2 transformed.

CHAPTER 5: DISCUSSION

Our first study revealed that, while no case-control divergence in serum anti-bacterial antibody concentrations was observed in RA patients prior to a diagnosis of RA compared to controls, a significant association was identified prior to RA diagnosis between anti-*P. intermedia* and anti-CCP2, ACPA fine specificities targeting vimentin, histone, and alpha-enolase, and IgA, IgG, and IgM RF autoantibody concentrations. In our second study, PD and periodontal clinical measures were not associated with serum anti-MAA antibody concentrations in the overall study population. However, ABL in RA cases, but not in OA controls, was significantly positively associated with IgG and IgM anti-MAA, underscoring a potential immunological bridge between the periodontium and RA. Furthermore, serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations were significantly positively associated with serum anti-MAA antibody concentrations in the entire study population (see Figure 1 below).



Postulated link between tissue inflammation, anti-MAA antibody formation and bone loss: A) inflammation in periodontitis or synovitis causes B) oxidative stress and C) MAA formation. Highly immunogenic, MAA-modified proteins lead to D) the formation of anti-MAA antibodies, which promote E) osteoclastogenesis and bone loss. Nucleases from *P. intermedia* degrade NETs leading to the release of PADs, generation of citrullinated antigens and ACPAs, which likewise induce osteoclastogenesis and bone loss. Abbreviations: ACPA, anti–citrullinated protein antibody; MAA, malondialdehyde-acetaldehyde; NET, neutrophil extracellular trap; PAD, peptidylarginine deiminase.

Relating the Findings to Current Understanding of Periodontitis and Rheumatoid Arthritis

This research explores the relationship between serum antibodies against *P. gingivalis, P. intermedia,* and *F. nucleatum* with RA diagnosis. Within chronically-inflamed periodontal tissues, the red complex, consisting of *P. gingivalis, T. forsythia,* and *T. denticola* (124), can induce a robust chronic inflammatory host response and potentially exacerbate RA (199). Beyond their effect on systemic inflammation, periodontal pathogens may also have a direct pathogenic role in RA. This is supported by the discovery of bacterial DNA in the synovial fluid in both native and failed prosthetic joints of patients with RA and OA, signifying translocation of periodontal bacteria from the periodontal tissues to the synovium resulting in amplified inflammatory processes (14). Our study demonstrated an absence of case-control divergence in anti-bacterial antibody concentrations in RA patients prior to RA onset, similar to an observation made by Manoil et al. (173). While the longitudinal elevations of these serum antibacterial antibodies alone were not observed prior to RA diagnosis, the observation that serum anti-*P. intermedia* was positively and significantly associated with RA autoantibody concentrations prior to RA diagnosis suggests a potential contribution of this organism to RA pathogenesis. This observation aligns with the concept that RA is a multifactorial process of host and environmental factors which affect the risk of disease onset and severity (200).

While previous studies have primarily focused on *P. gingivalis* due to its unique PAD activity, our study highlights the potential contributory role of *P. intermedia* in RA pathogenesis. A noteworthy finding of our study was a significant association between serum anti-*P. intermedia* antibodies and RA autoantibody concentrations prior to RA diagnosis, as specified above. *P. intermedia*, through mechanisms such as the expression of nucleases and its pronounced DNA degradation activity, as discussed by Doke et al., may contribute to immune evasion strategies, compromising the host's antimicrobial defenses (125). Furthermore, the role of these antibodies in facilitating the breakdown of immune tolerance presents a compelling narrative on how oral

dysbiosis by periodontal pathogens can cultivate a persistent state of inflammation, potentially leading in the development of autoimmunity (201).

MAA adducts have been linked to various inflammatory diseases (18, 184) including increased MAA-modified proteins in the synovium of RA patients (18) and inflamed human periodontal tissue as an extrasynovial source of MAA (21). Our research has identified a significant and positive association between serum anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations with serum anti-MAA antibody concentrations in the entire study population. This finding suggests the immune response to periodontal pathogens extends beyond local infection to trigger systemic inflammatory pathways that intertwine MAA production and RA.

A study by Akhi et al. proposed saliva IgA antibodies to MAA-LDL cross-reacted with *P. gingivalis*, attributing this observation to molecular mimicry (92). Additionally, Sherina et al. hypothesized *P. gingivalis* triggers loss of citrulline-tolerance through potential cross-reactivity with anti-*P. gingivalis* antibodies and human citrullinated proteins by mechanisms of molecular mimicry (7). Molecular mimicry refers to a situation where similarities between foreign and self-peptides favor an activation of autoreactive T or B cells by foreign-derived peptides, leading the immune system to mistakenly attack the host's own tissues. This mechanism is particularly relevant in the context of autoimmune diseases, such as RA (202). Although there is a scarcity of evidence tying certain autoimmunity mechanisms to PD, this does not negate their potential involvement in PD pathogenesis. Considering the role of these mechanisms in other inflammatory and autoimmune conditions, one study proposed that autoreactive cells in periodontal tissues may not directly contribute to tissue loss but could instead signify the activation of mechanisms aimed at controlling damage (201). This is supported by the observation that these mechanisms are also activated in periodontally healthy individuals. Nonetheless, the translocation of oral bacteria, their components, or metabolites from periodontal sites may play a role in systemic autoimmune reactions, although the precise nature of this relationship remains to be fully revealed. Therefore,

the connection between autoimmunity and periodontal pathogens at a systemic level should be understood in the context of dysbiosis leading to systemic autoimmune responses (201).

Our findings feature the role of periodontal pathogens in systemic inflammation, but they also showcased a lack of association between PD and periodontal clinical measures with serum anti-MAA antibody concentration. This finding underscores the complexity of the interaction between the periodontium and systemic inflammatory responses. While MAA may not directly correlate with the localized clinical manifestations of PD, MAA is intrinsically linked to systemic inflammatory responses (203) and bone resorption (204), both key factors in RA pathogenesis. Furthermore, our findings notably revealed ABL in RA cases, not in OA controls, was significantly and positively associated with serum IgG and IgM anti-MAA, suggesting a potential immunological link between the periodontium and RA. This observation is supported by research from Sakuraba et al. who reported that anti-MDA antibodies, which target a similar predominant epitope as anti-MAA antibodies, play a role in osteoclast differentiation and bone erosion (72). Anti-MDA antibodies induce significant metabolic changes in osteoclast precursors, notably boosting glycolysis and oxidative phosphorylation, resulting in increased adenosine triphosphate production. This study emphasizes the crucial role of lipid biosynthesis in osteoclast development, highlighted by the accumulation of citrate, a lipid precursor, and the impact of glycerolipid biosynthesis inhibitors on suppressing osteoclast maturation. The study reveals that anti-MDA antibodies promote lipid biosynthesis, likely through increased citrate synthesis and the transcriptional regulation of enzymes in the glycerolipid pathway. This represents a novel immune-complex mediated regulatory mechanism in osteoclastogenesis that might contribute to RA-associated bone erosion (72).

Taken together, our studies add further to the understanding of the relationship between PD and RA, offering insight into how *P. gingivalis*, *P. intermedia*, and *F. nucleatum* could influence the development and progression of RA beyond the oral cavity. The periodontal pathogens were also associated with anti-MAA in RA cases and OA controls, emphasizing a broader impact of

periodontal infection. This study supports MAA's role in systemic inflammation and bone resorption in RA patients. Overall, the results highlight the potential role of periodontal pathogens in systemic inflammation and RA onset and also suggest the potential of anti-MAA antibodies as biomarkers for RA among individuals with periodontal disease. Such insights emphasize a new era of integrated care that bridges dental care and systemic disease management.

Clinical Implications and Therapeutic Strategies

The findings from our research underscore a crucial link between PD and RA, shedding light on the potential of *P. gingivalis*, *P. intermedia*, and *F. nucleatum* in initiating systemic inflammation. Anti-*P. intermedia* antibodies were specifically associated with RA autoantibodies prior to RA onset during the preclinical period, highlighting the potential of periodontal pathogens to serve as early indicators for RA development. Healthcare providers, particularly rheumatologists and dentists, should consider periodontal screening and management as integral components of RA treatment strategies as early detection and treatment of PD could mitigate the severity of RA (131). Additionally, our findings suggest ABL may serve as a potential mediator linking PD and RA, hinting at an osteoimmunologic connection that warrants further investigation.

Currently, there are no FDA-approved therapies for RA that prevent AA or MDA from initiating pathogenic effects or combining to form the immunogenic MAA adduct (205). However, an FDA-approved treatment known as an aldehyde trap, or reactive aldehyde species inhibitor (RASPi), exists for Sjögren-Larsson syndrome which can prevent aldehyde adduct formation (206). Preliminary results from our group’s ongoing study indicate that RASP can also bind to MAA in vitro (unpublished results), preventing immune cell activation and the release of proinflammatory cytokines. Since MAA contributes to pathological processes, RASPi could offer a therapeutic approach by neutralizing the effects of MAA or AA and reduce systemic inflammatory responses.

Overall, the insights gained from our work advocate for a more integrated approach to patient care, where oral health markers are considered pivotal in the early detection, intervention, and comprehensive management of RA. This strategy not only aims to alleviate the systemic impact of RA but also highlights the importance of advancing research into innovative therapeutic approaches to address the underlying causes of inflammation in RA.

Limitations and Future Directions in Research

There are limitations to our studies, including the cross-sectional design and the possibility of limited generalizability due to the military population in the first study and a cohort comprised primarily of U.S. veterans in the second study. There were a higher proportion of men to women in the first (52% vs. 48%, respectively) and second (63% versus 37%, respectively) studies. Additionally, the first study included individuals with a younger age of RA onset (37 years old), compared to most epidemiologic studies where the mean age of RA onset approaches 50 years old (175). Though the overall results may not be generalizable to a more typical RA population, multivariable adjustments in our study and prior work from others did not indicate meaningful associations of sex with the study outcomes examined. In light of these considerations, our finding should be interpreted with caution until these results are validated in independent study populations (175). Regarding the first study, it is possible that the actual onset of clinically-apparent synovitis may precede the recorded diagnosis date due to delays in individuals seeking medical attention for joint symptoms. This delay could lead to an overestimation of the time span between the elevation of autoantibodies and the onset of synovitis. However, considering that the median time from clinically-apparent synovitis and RA prior to their recorded diagnosis was less than six months, as reported by Kelmenson et al., this factor may not significantly confound our findings (170).

In refining the first study, we propose several enhancements that could deepen the understanding of the interplay between serum antibodies to periodontal pathogens and the onset

of RA in future studies. By increasing the frequency of preclinical serum sample collection, a more precise temporal association between the presence of antibacterial antibodies and the development of RA could be assessed. A longitudinal study design would allow for observations regarding the evolution of antibody levels against periodontal pathogens and their association with RA-specific autoantibodies and disease onset, thereby providing stronger evidence for causality. Furthermore, expanding the participant pool to reflect the typical RA demographic more accurately can enhance the generalizability of the findings.

Given the results of our two studies, there is a need for a more clinical-translational and basic science investigation to uncover the mechanisms underpinning the involvement of MAA in disease pathogenesis. A proposed in vitro study, for example, could explore the signaling pathways activated by MAA in immune cells, such as macrophages, dendritic cells and T cells, to understand how MAA contributes to the autoimmune response in RA. Additionally, longitudinal assessments which include more frequent serum sampling throughout the study period would provide more detailed data on the temporal dynamics of anti-bacterial and anti-MAA antibody concentrations during the preclinical evolution of RA. Future MAA studies also should incorporate longitudinal designs to elucidate the temporal relationship between loss of alveolar bone over time and elevations of serum anti-MAA antibodies. This study design would provide information on causality and directionality of the RA/PD association, and investigate the specific biological mechanisms involved.

Modified HSA was used in the anti-MAA assay in our study. Incorporating a broader range of RA-relevant antigens (e.g., MAA-modified vimentin, fibrinogen, or type II collagen) could improve the performance of the anti-MAA assay, a possibility under active investigation. To improve periodontal diagnosis accuracy, utilizing vertical bitewings alongside comprehensive periodontal examinations is highly recommended over panoramic radiographs, which were used due to enhanced feasibility across study sites in the study described in Chapter 4. Incorporating the aforementioned design modifications could lead to a better understanding of the RA/PD

relationship, thereby enhancing patient care and treatment paradigms in the context of both diseases.

CHAPTER 6: CONCLUSION

Our studies shed light on the intricate relationships between PD, RA, periodontal pathogens, and MAA adduct formation, offering new insights into the interconnected PD-RA disease pathogenesis. Despite no observed differences in serum anti-bacterial antibody levels between RA patients and controls prior to RA diagnosis, our study uniquely identified a significant association between anti-*P. intermedia* antibodies and anti-CCP2, ACPA targeting specific proteins, and RF autoantibodies before RA onset. PD and periodontal clinical measures were not directly associated with serum anti-MAA antibody levels in the overall population in our second study, yet we identified a significant association between ABL in RA cases with IgG and IgM anti-MAA antibodies, suggesting that immune responses to MAA play a unique role in promoting RA disease progression that simultaneously promotes bone loss in PD. Furthermore, serum anti-*P. gingivalis,* anti-*P. intermedia,* and anti-*F. nucleatum* antibody concentrations were significantly positively associated with serum anti-MAA antibody concentrations in the entire study population. These findings underscore the multifactorial interplay between the periodontium and RA. Future studies should aim to further clarify the temporal relationships and causal mechanisms underlying these associations, potentially leading to targeted therapies and preventive strategies.

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